

09/831577

JCD5 Rec'd PCT/PTO 09 MAY 2001

Practitioner's Docket No. 6242

CHAPTER II

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.' " M.P.E.P., § 601, 7th ed.

TRANSMITTAL LETTER  
TO THE UNITED STATES ELECTED OFFICE (EO/US)

(ENTRY INTO U.S. NATIONAL PHASE UNDER CHAPTER II)

PCT/IT99/00355	8 November 1999	9 November 1998
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
METHOD TO CONTROL GENE EXPRESSION IN BACTERIA, NAMELY RHIZOBIACEAE		
TITLE OF INVENTION TO IMPROVE ROOT NODULE DEVELOPMENT, NITROGEN FIXATION, AND PLANT BIOMASS PRODUCTION		
APPLICANT(S)		
Roberto Defez and Angelo Spena		

Box PCT

Assistant Commissioner for Patents  
Washington D.C. 20231

ATTENTION: EO/US

CERTIFICATION UNDER 37 C.F.R. § 1.10\*

(Express Mail label number is mandatory.)

(Express Mail certification is optional.)

I hereby certify that this Transmittal Letter and the papers indicated as being transmitted therewith is being deposited with the United States Postal Service on this date \_\_\_\_\_, in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL826361900US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Sarah Kennedy

(type or print name of person mailing paper)

Signature of person mailing paper

**WARNING:** Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.

**\*WARNING:** Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).

"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

09/831577  
JC08 Rec'd PCT/PTO 09 MAY 2001

**NOTE:** To avoid abandonment of the application, the applicant shall furnish to the USPTO, not later than 20 months from the priority date: (1) a copy of the international application, unless it has been previously communicated by the International Bureau or unless it was originally filed in the USPTO; and (2) the basic national fee (see 37 C.F.R. § 1.492(a)). The 30-month time limit may not be extended. 37 C.F.R. § 1.495.

**WARNING:** Where the items are those which can be submitted to complete the entry of the international application into the national phase are subsequent to 30 months from the priority date the application is still considered to be in the international state and if mailing procedures are utilized to obtain a date the express mail procedure of 37 C.F.R. § 1.10 must be used (since international application papers are not covered by an ordinary certificate of mailing—See 37 C.F.R. § 1.8).

**NOTE:** Documents and fees must be clearly identified as a submission to enter the national state under 35 U.S.C. § 371 otherwise the submission will be considered as being made under 35 U.S.C. § 111. 37 C.F.R. § 1.494(f).

**I.** Applicant herewith submits to the United States Elected Office (EO/US) the following items under 35 U.S.C. § 371:

- a.  This express request to immediately begin national examination procedures (35 U.S.C. § 371(f)).
- b.  The U.S. National Fee (35 U.S.C. § 371(c)(1)) and other fees (37 C.F.R. § 1.492) as indicated below:

## 2. Fees

JC08 Rec'd PCT/PTO 09 MAY 2007  
09/831577

CLAIMS FEE	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
<input checked="" type="checkbox"/>	<b>TOTAL CLAIMS</b>	21 -20 =	1	$\times \$18.00 =$	\$ 18.00
	<b>INDEPENDENT CLAIMS</b>	1 -3 =	0	$\times \$80.00 =$	0.00
	<b>MULTIPLE DEPENDENT CLAIM(S) (if applicable)</b>			+ \$270.00	
<b>BASIC FEE**</b>	<input type="checkbox"/> <b>U.S. PTO WAS INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY</b> Where an International preliminary examination fee as set forth in § 1.482 has been paid on the international application to the U.S. PTO: <input type="checkbox"/> and the international preliminary examination report states that the criteria of novelty, inventive step (non-obviousness) and industrial activity, as defined in PCT Article 33(1) to (4) have been satisfied for all the claims presented in the application entering the national stage (37 C.F.R. § 1.492(a)(4)) ..... \$100.00 <input type="checkbox"/> and the above requirements are not met (37 C.F.R. § 1.492(a)(1)) ..... \$690.00 <input checked="" type="checkbox"/> <b>U.S. PTO WAS NOT INTERNATIONAL PRELIMINARY EXAMINATION AUTHORITY</b> Where no international preliminary examination fee as set forth in § 1.482 has been paid to the U.S. PTO, and payment of an international search fee as set forth in § 1.445(a)(2) to the U.S. PTO: <input type="checkbox"/> has been paid (37 C.F.R. § 1.492(a)(2)) ..... \$710.00 <input type="checkbox"/> has not been paid (37 C.F.R. § 1.492(a)(3)) ..... \$1000.00 <input checked="" type="checkbox"/> where a search report on the international application has been prepared by the European Patent Office or the Japanese Patent Office (37 C.F.R. § 1.492(a)(5)) ..... \$860.00				
	<b>Total of above Calculations</b>		=	\$ 878.00	
<b>SMALL ENTITY</b>	<b>Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (note 37 C.F.R. § 1.9, 1.27, 1.28)</b>			-	
	<b>Subtotal</b>			\$ 878.00	
	<b>Total National Fee</b>		\$	\$ 878.00	
	<b>Fee for recording the enclosed assignment document \$40.00 (37 C.F.R. § 1.21(h)). (See Item 13 below). See attached "ASSIGNMENT COVER SHEET".</b>				
<b>TOTAL</b>	<b>Total Fees enclosed</b>			\$ 878.00	

09/831577

JC08 Rec'd PCT/PTO 09 MAY 2001

\*See attached Preliminary Amendment Reducing the Number of Claims.

- Attached is a  check  money order in the amount of \$ 878.00
- Authorization is hereby made to charge the amount of \$ \_\_\_\_\_
  - to Deposit Account No. 19-0079
  - to Credit card as shown on the attached credit card information authorization form PTO-2038.

**WARNING:** Credit card information should not be included on this form as it may become public.

- Charge any additional fees required by this paper or credit any overpayment in the manner authorized above.

A duplicate of this paper is attached.

**\*\*WARNING:** "To avoid abandonment of the application the applicant shall furnish to the United States Patent and Trademark Office not later than the expiration of 30 months from the priority date: \* \* \* (2) the basic national fee (see § 1.492(a)). The 30-month time limit may not be extended." 37 C.F.R. § 1.495(b).

**WARNING:** If the translation of the international application and/or the oath or declaration have not been submitted by the applicant within thirty (30) months from the priority date, such requirements may be met within a time period set by the Office. 37 C.F.R. § 1.495(b)(2). The payment of the surcharge set forth in § 1.492(e) is required as a condition for accepting the oath or declaration later than thirty (30) months after the priority date. The payment of the processing fee set forth in § 1.492(f) is required for acceptance of an English translation later than thirty (30) months after the priority date. Failure to comply with these requirements will result in abandonment of the application. The provisions of § 1.136 apply to the period which is set. Notice of Jan. 3, 1993, 1147 O.G. 29 to 40.

**3.  A copy of the International application as filed (35 U.S.C. § 371(c)(2)):**

**NOTE:** Section 1.495 (b) was amended to require that the basic national fee and a copy of the international application must be filed with the Office by 30 months from the priority date to avoid abandonment. "The International Bureau normally provides the copy of the international application to the Office in accordance with PCT Article 20. At the same time, the International Bureau notifies applicant of the communication to the Office. In accordance with PCT Rule 47.1, that notice shall be accepted by all designated offices as conclusive evidence that the communication has duly taken place. Thus, if the applicant desires to enter the national stage, the applicant normally need only check to be sure the notice from the International Bureau has been received and then pay the basic national fee by 30 months from the priority date." Notice of Jan. 7, 1993, 1147 O.G. 29 to 40, at 35-36. See item 14c below.

- a.  is transmitted herewith.
- b.  is not required, as the application was filed with the United States Receiving Office.
- c.  has been transmitted
  - i.  by the International Bureau.

Date of mailing of the application (from form PCT/1B/308):  
\_\_\_\_\_

ii.  by applicant on \_\_\_\_\_. (Date)

**4.  A translation of the International application into the English language (35 U.S.C. § 371(c)(2)):**

- a.  is transmitted herewith.
- b.  is not required as the application was filed in English.
- c.  was previously transmitted by applicant on \_\_\_\_\_. (Date)
- d.  will follow.

09/831577

JC08 Rec'd PCT/PTO 09 MAY 2001

5.  Amendments to the claims of the International application under PCT Article 19  
(35 U.S.C. § 371(c)(3)):

NOTE: *The Notice of January 7, 1993 points out that 37 C.F.R. § 1.495(a) was amended to clarify the existing and continuing practice that PCT Article 19 amendments must be submitted by 30 months from the priority date and this deadline may not be extended. The Notice further advises that: "The failure to do so will not result in loss of the subject matter of the PCT Article 19 amendments. Applicant may submit that subject matter in a preliminary amendment filed under section 1.121. In many cases, filing an amendment under section 1.121 is preferable since grammatical or idiomatic errors may be corrected."* 1147 O.G. 29-40, at 36.

- a.  are transmitted herewith.
- b.  have been transmitted
  - i.  by the International Bureau.  
Date of mailing of the amendment (from form PCT/1B/308):  
18 May 2000
  - ii.  by applicant on \_\_\_\_\_ (Date)
- c.  have not been transmitted as
  - i.  applicant chose not to make amendments under PCT Article 19.  
Date of mailing of Search Report (from form PCT/ISA/210.):  
\_\_\_\_\_
  - ii.  the time limit for the submission of amendments has not yet expired.  
The amendments or a statement that amendments have not been made will be transmitted before the expiration of the time limit under PCT Rule 46.1.

6.  A translation of the amendments to the claims under PCT Article 19  
(38 U.S.C. § 371(c)(3)):
- a.  is transmitted herewith.
  - b.  is not required as the amendments were made in the English language.
  - c.  has not been transmitted for reasons indicated at point 5(c) above.
7.  A copy of the international examination report (PCT/IPEA/409)
  - is transmitted herewith.
  - is not required as the application was filed with the United States Receiving Office.
8.  Annex(es) to the international preliminary examination report
  - a.  is/are transmitted herewith.
  - b.  is/are not required as the application was filed with the United States Receiving Office.
9.  A translation of the annexes to the international preliminary examination report
  - a.  is transmitted herewith.
  - b.  is not required as the annexes are in the English language.

(Transmittal Letter to the United States Elected Office (EO/US) [13-18]—page 5 of 8)

09/831577

JC08 Rec'd PCT/PTO 09 MAY 2001

10.  An oath or declaration of the inventor (35 U.S.C. § 371(c)(4)) complying with 35 U.S.C. § 115
- a.  was previously submitted by applicant on \_\_\_\_\_ Date
  - b.  is submitted herewith, and such oath or declaration
    - i.  is attached to the application.
    - ii.  identifies the application and any amendments under PCT Article 19 that were transmitted as stated in points 3(b) or 3(c) and 5(b); and states that they were reviewed by the inventor as required by 37 C.F.R. § 1.70.
  - c.  will follow.

**II. Other document(s) or information included:**

11.  An International Search Report (PCT/ISA/210) or Declaration under PCT Article 17(2)(a):
- a.  is transmitted herewith.
  - b.  has been transmitted by the International Bureau.  
Date of mailing (from form PCT/IB/308): \_\_\_\_\_
  - c.  is not required, as the application was searched by the United States International Searching Authority.
  - d.  will be transmitted promptly upon request.
  - e.  has been submitted by applicant on \_\_\_\_\_ Date
12.  An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98:
- a.  is transmitted herewith.  
Also transmitted herewith is/are:
    - Form PTO-1449 (PTO/SB/08A and 08B).
    - Copies of citations listed.
  - b.  will be transmitted within THREE MONTHS of the date of submission of requirements under 35 U.S.C. § 371(c).
  - c.  was previously submitted by applicant on \_\_\_\_\_ Date
13.  An assignment document is transmitted herewith for recording.

A separate  "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or  FORM PTO 1595 is also attached.

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

09/831577

JC08 Rec'd PCT/PTO 09 MAY 2001

14.  Additional documents:

- a.  Copy of request (PCT/RO/101)
- b.  International Publication No. WO 00/28051
  - i.  Specification, claims and drawing
  - ii.  Front page only
- c.  Preliminary amendment (37 C.F.R. § 1.121)
- d.  Other
  - Form PCT/IB/304; Form PCT/IB/308; Form PCT/IPEA/402;
  - Form PCT/IB/332; Submission of "Sequence Listing" computer
  - readable copy and statement for biotechnology invention
  - containing nucleotide and/or amino acid sequences.

15.  The above checked items are being transmitted

- a.  before 30 months from any claimed priority date.
- b.  after 30 months.

16.  Certain requirements under 35 U.S.C. § 371 were previously submitted by the applicant on \_\_\_\_\_, namely:

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### AUTHORIZATION TO CHARGE ADDITIONAL FEES

**WARNING:** Accurately count claims, especially multiple dependant claims, to avoid unexpected high charges if extra claims are authorized.

**NOTE:** "A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

**NOTE:** "Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

Please charge, in the manner authorized above, the following additional fees that may be required by this paper and during the entire pendency of this application:

- 37 C.F.R. § 1.492(a)(1), (2), (3), and (4) (filing fees)

**WARNING:** Because failure to pay the national fee within 30 months without extension (37 C.F.R. § 1.495(b)(2)) results in abandonment of the application, it would be best to always check the above box.

(Transmittal Letter to the United States Elected Office (EO/US) [13-18]—page 7 of 8)

09/831577

JC08 Rec'd PCT/PTO 09 MAY 2007

37 C.F.R. § 1.492(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.492(d)), it might be best not to authorize the PTO to charge additional claim fees, except possible when dealing with amendments after final action.

- 37 C.F.R. § 1.17 (application processing fees)  
 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).  
 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying . . . issue fee." From the wording of 37 C.F.R. § 1.28(b): (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

- 37 C.F.R. § 1.492(e) and (f) (surcharge fees for filing the declaration and/or filing an English translation of an International Application later than 30 months after the priority date).



SIGNATURE OF PRACTITIONER

Reg. No.: 35,985

Arlene J. Powers

Tel. No.: (617) 426-9180  
ext.: 110

(type or print name of practitioner)

Customer No.:

225 Franklin Street, Suite 3300

P.O. Address

Boston, MA 02110

09/831577  
6242

JC08 Rec'd PCT/PTO 09 MAY 2007

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANT: Roberto Defez et al.

GROUP: Unknown

SERIAL NO: Unknown

EXAMINER: Unknown

FILED: Herewith

FOR:

Assistant Commissioner of Patents

Washington, D.C. 20231

Sir:

**PRELIMINARY AMENDMENT**

Preliminary to examination, please amend the above-identified application as follows:

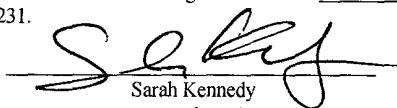
**IN THE CLAIMS:**

Please amend the claims as follows:

1        12. (Amended) The recombinant DNA molecule according to claim 8 wherein said DNA  
2        coding sequence encodes [both] the iaaM protein from P. syringae subsp. savastanoi or an  
3        homologous thereof and the tms2 protein from A. tumefaciens or an homologous thereof [coding  
4        regions of claim 10 and 11,] respectively.

1        14. (Amended) Genetically engineered bacteria comprising the recombinant DNA  
2        molecule according to [claims from 6 to 13] claim 6.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited on the date shown below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL826361900US addressed to the: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

  
Sarah Kennedy  
Date. 5/9/01

1        15. (Amended) Use of the recombinant DNA molecule according to [claims from 6 to 13]

2        claim 6 to significantly increase the size of nodules of a plant.

1        17. (Amended) Use of the recombinant DNA molecule according to [claims from 6 to 13]

2        claim 6 to significantly increase the capacity to fix nitrogen of a nodulated plant.

1        19. (Amended) Use of the recombinant DNA molecule according to [claims from 6 to 13]

2        claim 6 to significantly increase the plant biomass production.

1        21. (Amended) Legume plant infected by bacteria [harbouring] harboring the recombinant

2        DNA molecule according to [claims from 6 to 13] claim 6 and having a significant increase of the

3        size of nodules, and/or of the nodule capacity to fix nitrogen, and/or of the plant biomass, and/or

4        of the ability to fix nitrogen.

**REMARKS**

The present preliminary amendment is submitted in order to correct the improper multiple dependency of claims as originally filed.

Examination on the merits is respectfully requested.

Respectfully submitted,

  
Arlene J. Powers

Registration No.

Samuels, Gauthier & Stevens  
225 Franklin Street, Suite 3300  
Boston, Massachusetts 02110  
Telephone: (617) 426-9180  
Extension 110

09/831577

CLEAN COPY OF AMENDED CLAIMS 108 Rec'd PCT/PTO 09 MAY 2001

1        1. Use of the promintron sequence of the *rolA* gene from *Agrobacterium rhizogenes* as  
2        in SEQ ID NO. 1, or of DNA sequences comprising said promintron sequence, or of  
3        functional homologous or portion thereof, to induce the expression of a DNA coding sequence,  
4        in recombinant bacteria during exponential, post-exponential and stationary phase of growth,  
5        and in bacteroids within root nodules, said coding DNA sequence being under the control of  
6        said promintron sequence.

1        2. Use of the promintron sequence according to claim 1 wherein said recombinant  
2        bacteria belong to either the *Enterobacteriaceae* or the *Rhizobiaceae* families.

1        3. Use of the promintron sequence according to claim 2 wherein said recombinant  
2        bacteria belonging to either the *Enterbacteriaceae* or the *Rhizobiaceae* families are *E. coli*,  
3        *Rhizobia* or *Agrobacteria*.

1        4. Use of the promintron sequence according to claim 3 wherein said recombinant  
2        bacteria are of the *Rhizobia* genus, either within symbiotic root nodules or in a free living  
3        status.

1        5. Use of the promintron sequence according to claim 4 wherein said recombinant  
2        bacteria of the *Rhizobia* genus within symbiotic root nodule, are either bacteroids of stage I, II,  
3        III, IV, V, or *Rhizobia* present in the apoplastic space, or *Rhizobia* present in the senescence  
4        zone, or *Rhizobia* present in the nitrogen fixing zone, or *Rhizobia* present in the invasion zone.

1       6. A recombinant DNA molecule comprising the promintron sequence according to  
2 claim 1, or functional homologous or portion thereof, and covalently linked to the 3' end of  
3 said promintron sequence, a DNA coding sequence, said recombinant DNA molecule being  
4 either harboured by prokaryotic episomal elements, or integrated in a bacterial genome.

1       7. The recombinant DNA molecule according to claim 6 wherein said DNA coding  
2 sequence is either a monocistronic or a polycistronic transcriptional unit.

1       8. The recombinant DNA molecule according to claim 7 wherein said DNA coding  
2 sequence encodes a protein involved in plant hormone auxin synthesis and/or metabolism.

1       9. The recombinant DNA molecule according to claim 8 wherein said DNA coding  
2 sequence encodes a protein involved in the synthesis and/or metabolism of the auxin IAA or of  
3 the auxin indolethanol.

1       10. The recombinant DNA molecule according to claim 8 wherein said DNA coding  
2 sequence encodes the *iaaM* protein from *P. syringae* subsp. *savastanoi* or an homologous  
3 thereof.

1       11. The recombinant DNA molecule according to claim 8 wherein said DNA coding  
2 sequence encodes the *tms2* protein from *A. tumefaciens* or an homologous thereof.

1       12. (Amended) The recombinant DNA molecule according to claim 8 wherein said  
2 DNA coding sequence encodes the *iaaM* protein from *P. syringae* subsp. *savastanoi* or an  
3 homologous thereof and the *tms2* protein from *A. tumefaciens* or an homologous thereof  
4 respectively.

1       13. The recombinant DNA molecule according to claim 8 wherein said DNA coding  
2 sequence encodes the indolepyruvate decarboxylase from *Enterobacter cloacae* or an  
3 homologous thereof.

1       14. (Amended) Genetically engineered bacteria comprising the recombinant DNA  
2 molecule according to claim 6.

1       15. (Amended) Use of the recombinant DNA molecule according to claim 6 to  
2 significantly increase the size of nodules of a plant.

1       16. Use of the recombinant DNA molecule according to claim 15 wherein said  
2 statistically significant increase of the nodule size is of at least 20%.

1       17. (Amended) Use of the recombinant DNA molecule according to claim 6 to  
2 significantly increase the capacity to fix nitrogen of a nodulated plant.

1       18. Use of the recombinant DNA molecule according to claim 17 wherein said  
2 statistically significant increase of the capacity to fix nitrogen is of at least 20%.

1       19. (Amended) Use of the recombinant DNA molecule according to claim 6 to  
2 significantly increase the plant biomass production.

1       20. Use of the recombinant DNA molecule according to claim 19 wherein said  
2 statistically significant increase of the plant biomass production is of at least 10%.

1       21. (Amended) Legume plant infected by bacteria harboring the recombinant DNA  
2 molecule according to claim 6 and having a significant increase of the size of nodules, and/or

- 3 of the nodule capacity to fix nitrogen, and/or of the plant biomass, and/or of the ability to fix
- 4 nitrogen.

09/831577

JC08 Res'd PCT/PTO 09 MAY 2001

12/PRTS

METHOD TO CONTROL GENE EXPRESSION IN BACTERIA, NAMELY *RHIZOBIACEAE*,  
TO IMPROVE ROOT NODULE DEVELOPMENT, NITROGEN FIXATION AND PLANT  
BIOMASS PRODUCTION.

DESCRIPTION

5       The present invention relates to the use of the  
promintron DNA sequence of the *rolA* gene of *Agrobacterium*  
*rhizogenes* strain A4, or of promintron DNA sequences  
derived thereof, or of promintron DNA sequences of  
homologous genes from other strains of *Agrobacterium*  
10      *rhizogenes*, for promoting gene expression at the  
transcriptional level in recombinant bacteria and to  
achieve, for example, synthesis of phytohormones within  
bacteria and/or bacteroids.

15      The present invention demonstrates that the *iaaM*  
gene of *Pseudomonas syringae* and the *tms2* gene of  
*Agrobacterium tumefaciens* are able to improve the  
nodulation process and nitrogen fixation when expressed  
as bicistronic unit in *Rhizobia* under the control of the  
promintron sequence. The promintron is particularly  
suited and useful for the use to drive gene expression  
within bacteroids in all stages of nodule development in  
order to obtain, over the developmental time of the  
nodule, a constitutive expression of the gene(s) of  
20      interest. Therefore, constitutive here means that, by  
different expression patterns at various stages of nodule  
development, a gene expression in all parts of the nodule  
where plant cells harbour *Rhizobia* is achieved.

25      The invention stems from the undisclosed  
demonstration that the 85 bp intron sequence of the *rolA*  
30      gene of *Agrobacterium* strain A4 is able to promote gene  
expression in prokaryotic cells. Therefore, the 85 bp DNA  
sequence has been named "promintron", indicating its  
function of intron in eucaryotic cells (Magrelli, A. et  
al. (1994) Science, 266, 1986-1988), and of promoter in  
35      prokaryotic cells. The term "promoter" refers to

nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding, and also includes for example the -35 and -10 boxes, or other regulatory regions.

5 The promoter activity in *Rhizobia* of the *rolA* promintron resulted to be able to get gene expression in a novel and peculiar pattern within root nodules, resulting in a constitutive expression.

10 The invention is particularly useful: i) to alter nodule development and to increase nitrogen fixation by either nodulating plants and/or by nitrogen fixing bacteria; ii) to improve plant growth and to accelerate plant biomass production by inoculating leguminous plants with genetically modified *Rhizobia* (*Rhizobia* is here used 15 as a collective name including *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Bradyrhizobium*).

20 The present invention also relates to DNA constructs in which said promintron controls the expression of a DNA sequence, which upon expression leads to the above mentioned effects. Furthermore, the present invention relates to genetically engineered bacteria which can improve nitrogen fixation and consequently, 25 plant biomass production. Furthermore, the improvement of nitrogen fixation in plants might be achieved in the presence of concentrations of nitrate negatively affecting the nodulation process.

30 The world population will most likely increase of 2 billions people in the next 25 years mostly in Asia and Africa. Proteins coming from legumes represent actually 33% of total human diet. Legume plants are able to self provide nitrogen through associations with nitrogen fixing bacteria of the genus *Rhizobia*, i.e. *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Bradyrhizobium*. Other plants do exist not belonging to 35 the *Leguminosae*, and yet able to host bacteria in a nitrogen-fixing nodule. A plant not belonging to the

leguminous family interacting with nitrogen fixing *Rhizobia* is *Parasponia* (Ulmaceae). Furthermore, actinomycetes of the genus *Frankia* interact with plant species from eight different families and cause the production of root nodules (Pawlowski, K. et al. (1998) in: Biological Nitrogen Fixation for the 21<sup>st</sup> Century, eds. C. Elmerich, A. Kondorosi and W.E. Newton, Kluwer Academic publisher, pp.199-201). Symbiotic nitrogen fixation plays a major role in the nitrogen cycle.

Nevertheless, agriculture exploitation results in a large uptake of organic nitrogen from the soil, which is compensated by providing plants with fertilisers. Nitrogen fertilisation however, provokes severe environmental problems because its excess contributes to water pollution. Nitrogen fertilisation represents one of the most important part of the farming costs. The world consumption of nitrogen fertilisers increased in the last 55 years from 3 to 100 TG ( $1\text{TG}=10^{12}\text{g}$ ). To produce nitrogen fertilisers, all over the world 2 to 5% of the not renewable energy sources (e.g. oil and other forms of fuel) are burned every year. An environmental problem associated with the use of synthetic nitrogen fertilisers is leakage of excess nitrate into the underground water. Up to 10% of the released nitrate ends as nitrous oxide, a greenhouse gas whose energy reflectivity amounts to 180 times that of carbon dioxide (Hardy, R.W.F. and Eaglesham, A.R.J. (1995). In: Nitrogen Fixation: Fundamentals and applications. I.A. Tikhonovich et al., (eds.), pp. 619-620). Many large towns in industrialised countries are presently facing the problem of nitrate pollution of drinkable water, and water price for human use will rapidly increase.

Therefore, the general problem underlying the present invention is represented by the need to improve the cultivation of leguminous and other nodulating plants, with the aim to contribute to a drastic decrease

of the nitrate release in the environment. A solution to these problems is either to increase the cultivation of plants able to fix atmospheric nitrogen or to increase their nitrogen fixation capacity.

5 Biological Nitrogen Fixation (BNF) by free-living, associative and symbiotic nitrogen-fixing microbes is responsible for the conversion of about 120 million tons  
10 of atmospheric nitrogen into ammonia every year, which represent 40% of the total nitrogen request (Vance, C.P., Third European Nitrogen Fixation Conference, Lunteren, The Netherlands, 1998, p. 17); symbiotic nitrogen fixation is the major naturally occurring mechanism by which nitrogen is reduced (Franssen, H.J. et al. (1992) Plant Molec. Biol., 19, 89-107). Members of the  
15 Rhizobiaceae family, which includes nitrogen-fixing *Rhizobia*, are able to enter into symbiosis with mainly leguminous plants, resulting in the formation of root nodules. Within the cells of the root nodule, the bacteria differentiate in bacteroids. Mature bacteroids  
20 fix atmospheric nitrogen and convert it into ammonia by a nitrogenase activity. The so-produced ammonium is exported from the nodules and is incorporated into plant amino acids, ureids and azotated bases. Thus, in the presence of a nodulated root, plant growth is independent  
25 of alternative nitrogen sources.

Root nodule development is the result of a symbiotic interaction controlled by several factors. Early phases of this interaction require the exchange of chemical signals between the two partners: the plant root  
30 and the bacteria. The plant root produces chemical signals, such as flavonoids, which attract the *Rhizobia*, and most importantly do induce the expression of bacterial genes, the *nod* genes, involved in the production of the so-called Nod factors. The Nod factors  
35 are lipooligosaccharides which play a crucial role in the early phases of nodule development (Denariè, J., Debelle,

F., and Promé, J.-C. (1996) Annual Review Biochemistry 65, 503-535).

While the role of the Nod factors in triggering root nodule development is well established, the role of phytohormones in root nodule development is still unclear. Phytohormones are plant growth factors able to modify plant growth and development. Since phytohormones are involved in almost any developmental and/or physiological process of the plant, a role of phytohormones in nodule development is most reasonable. However, despite efforts aimed to address the general question of a role of phytohormones in nodulation, in 1994 Hirsch and Fang summarised the state of the art with the following words: "Important questions remains unanswered: which hormones take part in nodule formation, and how are they involved?" (A.M. Hirsch and Y. Fang, (1994) Plant Mol. Biol. 26, 5-9).

Already in 1936, Thimann (Thimann, K. (1936) P.N.A.S. USA, 22, 511-514) proposed that auxin(s) plays a role in nodule development, and that root nodules do contain auxin(s). Furthermore, based on biological assays, Thimann showed that the auxin content, estimated as auxinic biological activity, within a nodule is higher than that one of uninfected roots.

Nowadays, it is well established that free living *Rhizobia* do contain auxins, such as indole-3-acetic acid (IAA), indole-3-ethanol, indole-3-aldehyde and indole-3-methanol (Ernstsen, A. et al. (1987) Planta 171, 422-428). Furthermore, flavonoids, i.e. chemical signals produced by the plant root which attract *Rhizobia* and stimulate the synthesis of Nod factors, stimulate IAA synthesis in *Rhizobia* grown in liquid culture (Prinsen, E. et al. (1991) FEBS Letters, 282, 53-55).

Although these data are consistent with a possible role of auxin(s) in nodule development, Hirsch in 1992 reported that "*Rhizobia* can synthesize plant hormones,

but mutations in their auxin-producing genes do not prevent nodulation, nor do Nod<sup>-</sup> mutants fail to produce hormones (Hirsch, A.M. (1992) New Phytologist, 122, 211-237).

5 Other studies addressing the "plant contribution" to the nodulation process, such as those of Hirsch, A.M. et al. (P.N.A.S. USA (1989) 86, 1244-1248), of Allen, E.K., Allen, O.N. and Newman, A.S. (Am. J. Botany (1953), 40, 429-435), and of Torrey, J.G. (in: "New root  
10 formation in plants and cuttings", 1986 ed. Jackson MB, Martinus Nijhoff Publishers, Dordrecht, 31-66), have found that inhibitors of polar auxin transport induced pseudonodules on alfaalfa roots. Mathesius et al. (The Plant Journal (1998) 14, 23-34) has recently shown that auxin transport inhibition precedes root nodule formation. Kijne, J.W. et al. (Third European Nitrogen fixation Conference, Lunteren, The Netherlands, 1998, p. 24), have shown that: i) a local increase of IAA precedes induction of cell divisions in the inner cortex and ii)  
15 nitrate inhibits the response to IAA rather than its accumulation.

Further indications of a role of auxinic phytohormones produced by the plant tissue in nodule development come from variants of *Medicago varia*, defined as auxin sensitive and usually containing a higher content of auxins, which formed more nodules than less sensitive variants (Kondorosi, E. et al. (1993) In: Nester, E.W. and Verma, D.P.S. eds., Advances in Molecular-Genetics of Plant-microbe interactions, pp.  
25 143-150). Furthermore, plants transgenic for the *rolB* gene nodulated faster and had more nodules (Kondorosi et al., ibidem). *RolB* is known to increase auxin sensitivity. The function of *rolB* is considered by some authors not to affect auxin content and to be a membrane receptor for the auxin IAA, whereas others (PCT  
30 Application No. WO 98/28430) consider the *RolB* protein a  
35

beta-glucosidase able to release the auxin indolethanol from indolethanol-glucoside. Indolethanol is considered to be an auxin per se, and it is converted to IAA, the major form of auxin in plants (Sembdner, G. et al. (1980) 5 in: *Encyclopedia of Plant Physiology*, vol. 9: Hormonal regulation of development. I. Molecular aspects of plant hormones, pp. 281-444; MacMillan, J. Ed., Springer, Berlin, Heidelberg, New York; Sandberg, G., Crozier, A., Ernstsen, A. (1987) In: *The Principles and practices of 10 plant hormone analysis*, vol. 2, pp. 169-301, Rivier, L., Crozier, A. eds. Academic Press, London).

The present invention has addressed three aspects related to the aforementioned problems: i) is it possible to modify root nodule development by expressing auxin synthesising genes within *Rhizobia*?; ii) has the plant nodulated by the genetically modified strain of *Rhizobia* an increased capacity to reduce atmospheric nitrogen to ammonia? iii) is the plant nodulated by the genetically modified *Rhizobia* altered in its growth, and is such alteration improving plant biomass production?

Genes coding for enzymes able to modify auxin metabolism can be taken from the genomes of plants, but also from the genomes of bacteria (Spina, A., Estruch, J.J. and Schell, J. (1992) *Current Opinion in 15 Biotechnology* 3, 159-163). Whatever the origin of the gene(s) and/or coding region(s), the proper specificity of expression within the bacteria and, most importantly, within the bacteroids must be conferred to the said 20 coding region(s) by positioning the coding region(s) under the control of a proper promoter (DNA) sequence.

The present invention solves the problems of the prior art by providing technical means to improve gene expression in recombinant bacteria, preferably *Rhizobiaceae*, and most preferably *Rhizobium*, and it is easily utilised to properly control the expression of 25 genes able to increase the auxin content and, as

consequence, to improve the nodulation and nitrogen fixation processes. The promintron sequence of the invention is able to promote prokaryotic expression in all the zones of the nodule where bacteroids are located  
5 with a temporally defined pattern of expression. Furthermore, the promintron drives gene expression during the exponential phase of growth and it is induced during the stationary phase of growth of free living bacteria. All these features are very advantageous when gene  
10 expression is to be promoted in nitrogen fixing soil bacteria under both exponential and stationary phase of growth, and in bacteroids within the root nodule.

In particular a gene construct of the invention comprises the promintron sequence of the *rolA* gene, as in SEQ ID No.1, and the coding regions of the *iaaM* from *Pseudomonas syringae* subsp. *savastanoi* and the *tms2* gene from *Agrobacterium tumefaciens*. The two coding regions have been built as bicistronic unit under the control of the *rolA* promintron, whose promoter activity takes place  
15 in the nodule in an undisclosed and novel pattern of expression. The combination of the promintron to the bicistronic unit, and its introduction in *Rhizobia*, has allowed the authors of the invention to show that auxin affects nodule development and function. The use of the  
20 two coding regions is meant to increase the synthesis and content of IAA, and consequently to increase also the synthesis and content of IAA conjugates (collective name indicating all the forms of IAA covalently conjugated either to amino acids or to glucose or to other chemical  
25 moieties) in the plant cells of the root nodule. The same effect might be achieved either by using the *iaaM* and *tms2* genes from other *Agrobacteria* or *Pseudomonas* strains, or by using other genes able to increase IAA  
30 content.

35 Since *Rhizobia* usually do not contain indoleacetamide, neither are able to convert tryptophane

to indoleacetamide (IAM), nor are able to convert IAM to IAA (Ernstsen et al., 1987, ibidem), the authors have engineered a novel biochemical pathway which requires both genes to efficiently synthesise IAA from tryptophane. However, in those *Rhizobia*, such as *Bradyrhizobium*, which do contain the *bam* gene - an endogenous homologue of the *tms2* gene (Sekine, M., Watanabe, K. and Syono, K. (1989) J. Bacteriol. 171, 1718-1724) - this might be sufficient to express only the *iaaM* gene under the control of the promintron DNA sequence. A functional homologous of *tms2* is also present in some higher plants as shown in the PCT Application WO98/28430.

In a further embodiment of the present invention the DNA sequence expression of which is driven by the promintron is one coding for indolepyruvate decarboxylase. Indolepyruvate decarboxilase is the key enzyme in the indolepyruvic pathway of IAA synthesis in which IAA is synthesised from tryptophane. The conversion of indolepyruvic acid to IAA is the rate limiting step of this pathway. A DNA sequence encoding a suitable indolepyruvate decarboxylase has been cloned, for example, from *Enterobacter cloacae* (see, e.g. Koga et al., Mol. Gen. Genet. (1991), 226, 10-16).

The term "auxin" comprises in this context naturally occurring and synthetic organic substances acting as phytohormones in the sense that they promote elongation of shoots and inhibit elongation of roots, preferably in low concentrations, most preferably already in concentrations lower than  $10^{-6}$  M. Preferably, an auxin shows at least one of the following effects on plant development: stimulation of cell division, of cell elongation, and/or of cell expansion, of apical dominance, stimulation of xylem differentiation, stimulation of the cell elongation and cell division activity of the cells of the cambium, stimulation of

lateral and adventitious root formation, stimulation of nodulation, of germination, of leaf epinasty, of ovary cell growth, of parthenocarpy, of the formation of female flowers and of leaf expansion. More particularly, the term "auxin" refers to indole 3-acetic acid (IAA) which is most likely synthesised in plant cells from tryptophane via indole-3-pyruvate and indole-3-acetaldehyde, and which is degraded via enzymatically catalysed oxidation. However, the term also comprises other naturally occurring compounds which act as an auxin and which are derived from indole or another compound, for example, the naturally occurring phenyl acetic acid which is a non-indolic auxin or 4-(indole-3-yl) butyric acid. Furthermore, this term comprises compounds from organisms other than plants or chemically synthesised compounds which have at least one of the effects on plant development as listed above. An example for such compound is (2,4-dichlorophenoxy)-acetic acid (2,4-D).

In a preferred embodiment the DNA sequence linked to the promintrone codes for a polypeptide which is naturally involved in the biosynthesis of at least one auxin. The expression of the DNA sequence in *Rhizobia* cells leads to an increase in the biological (for example, enzymatic) activity of this polypeptide and consequently to the increase of the content of at least one auxin first in the bacteroid, and after auxin export from the bacteroids, in plant cells of the nodule. Thus, in principle, by this embodiment it is contemplated that the auxin content and/or activity can be increased by increasing the biosynthesis of at least one auxin due to a stimulation/acceleration of a biosynthetic pathway leading to the synthesis of auxin. In another preferred embodiment the DNA sequence linked to the promintrone codes for a protein which is naturally not expressed in *Rhizobia* and which upon expression in bacteroids leads to the synthesis of at least one auxin or a precursor of an

auxin from a metabolite present in the bacterial cell and/or bacteroid. One example of a gene which is preferably used for the purpose of the present invention is the *iaaM* gene of *Pseudomonas syringae* subsp. *Savastanoi*, the ethiological agent of plant tumors in olive and oleander trees (Spina, A., Estruch, J.J., Schell, J., Curr. Opinion in Biotechnology (1992), 3, 159-163). The neoplastic development is caused by phytohormones synthesised by the bacteria, which are then secreted into the surrounding tissues and stimulate the localised growth of plant cells. Among the genes involved in the pathogenesis of this type of tumour, the *iaaM* gene codes for the indoleacetamide monooxygenase, and it is responsible for converting by oxidation the amino acid tryptophane to indoleacetamide. Indoleacetamide has no particular auxin activity, but it is converted to IAA by the hydrolase encoded by the *iaaH* gene of *Pseudomonas syringae* subsp. *Savastanoi*, which is homologue to the *tms2* gene of *Agrobacterium tumefaciens*. In plant tissues, indoleacetamide is also converted to IAA, either chemically or by unspecific hydrolases.

The *iaaM* gene from *Pseudomonas syringae* subsp. *Savastanoi* is known and its sequence has been published (Yamada et al. (1985) P.N.A.S. USA, 82, 6522-6526). The *tms2* gene from *Agrobacterium tumefaciens* is known and its sequence has been published (Klee, H. et al. (1984) P.N.A.S. USA 81, 1728-1732). According to the invention genes homologous in function to the *iaaM* gene of *P. syringae* and to the *tms2* gene of *Agrobacterium tumefaciens* might be used for the purpose of this invention. Such genes which are preferably also homologous with respect to the nucleotide sequence can be isolated by the person skilled in the art using known methods, e.g. the screening of cDNA or genomic libraries with probes designed on the basis of the *iaaM* gene of *Pseudomonas syringae* and/or *tms2* gene of *Agrobacterium*.

tumefaciens. Such genes with an activity similar to that of the *iaaM* gene product of *P. syringae* have been cloned, for instance, from some strains of *Agrobacteria* (i.e. *Agrobacterium tumefaciens* and *rhizogenes*; see, for instance: Klee et al. (1987), Gene Dev, 1, 186-196; White et al. (1985) J. Bacteriol., 164, 33-44; Cardarelli et al. (1987), Mol. Gen. Genet., 208, 457-463.

The 85 bp DNA promintron sequence of the *rolA* gene from Ri plasmid A4 (SEQ ID No.1) promotes gene expression of DNA sequences positioned under its control (i.e. linked to its 3' end) in prokaryotic systems, such as *E.coli*, *Rhizobia* and *Agrobacteria*, during exponential, post-exponential and stationary phases of growth. Furthermore, the promintron promotes gene expression in bacteroids within root nodules. The person skilled in the art may easily and with no inventive effort isolate promintron sequences from other strains of *Agrobacterium rhizogenes* and/or isolate functional homologue promoters, namely promoters driving gene expression with an identical pattern in bacteroids within root nodules and/or in free living bacteria.

It is therefore a specific object of the present invention the use of the promintron sequence of the *rolA* gene from *Agrobacterium rhizogenes* as in SEQ ID No. 1, or of DNA sequences comprising said promintron sequence, or of functional homologue or portion thereof, to induce the expression of a DNA coding sequence, in recombinant bacteria during exponential, post-exponential and stationary phase of growth, and in bacteroids within root nodules, said coding DNA sequence being under the control of said promintron sequence, or of functional homologous promoters. Preferably the recombinant bacteria belong to either the *Enterobacteriaceae* or the *Rhizobiaceae* families, more preferably are *E. coli*, *Rhizobia* or *Agrobacteria*.

In a preferred embodiment the recombinant bacteria are of the *Rhizobia* genus, either within symbiotic root nodules or in a free living status. When bacteria are within symbiotic root nodules, they are either bacteroids of stage I, II, III, IV, V, or *Rhizobia* present in the apoplastic space, or *Rhizobia* present in the senescence zone, or *Rhizobia* present in the nitrogen fixing zone, or *Rhizobia* present in the invasion zone.

It is further object of the invention a recombinant DNA molecule comprising the promintron sequence according to the invention, or functional homologue or portion thereof, and covalently linked to the 3' end of the promintron sequence, a DNA coding sequence, wherein the recombinant DNA molecule is either harboured by episomal elements or integrated in the bacterial genome. Preferably the DNA coding sequence is either a monocistronic or a polycistronic transcription unit, more preferably encodes a protein involved in plant hormone synthesis and/or metabolism, most preferably encodes a protein involved in plant hormone auxin synthesis and/or metabolism, most preferably encodes a protein involved in the synthesis and/or metabolism of the auxin IAA or of the auxin indolethanol, even more preferably encodes the *iaaM* protein from *P. syringae* subsp. *savastanoi* or an homologous (i.e. with a similar enzymatic activity) thereof and/or encodes the *tms2* protein from *A. tumefaciens* or an homologous thereof. In a preferred aspect the recombinant DNA molecule according to the invention comprise both the *iaaM* and the *tms2* coding regions.

Alternatively the recombinant DNA molecule of the invention comprises a DNA coding sequence encoding the indolepyruvate decarboxylase from *Enterobacter cloacae* or an homologous thereof.

It is a further object of the invention genetically engineered bacteria comprising the recombinant DNA

molecule described. Bacteria are preferably comprised in the following list: *Rhizobia*, as above specified, *Azotobacter*, *Azospirillum*, *Anabaena*, *Enterobacteriaceae*.

It is in the scope of the invention the use of the recombinant DNA molecule to increase nodule size and/or activity, and/or to increase plant biomass production.

It is in the scope of the invention the use of the recombinant DNA molecule to increase the capacity of nitrogen fixation of root nodules.

The invention will be described in some examples by reference to the following figures:

Figure 1: Schematic drawing of the chimeric reporter genes. The 86AGUS construct contains: i) an 86 bp long DNA fragment comprising the 85 bp long promintron sequence as shown in SEQ ID No. 1 plus one A residue at its 3'end; ii) the DNA sequence corresponding to the first 40 amino acids of the *rolA* protein fused to iii) the *uidA* coding region. The 85-17GUS construct contains 5' to 3' end: an Eco RI restriction site (GAATTC), the promintron sequence of SEQ ID No. 1, a 17 bp linker sequence (SEQ ID No. 2), a KpnI restriction site (GGTACC), before the ATG of the *uidA* coding region. Δ GUS indicates the construct containing just the *uidA* coding region, i.e. without any promintron sequence. Promintron indicates the 86 bp sequence comprising the 85 bp of the *rolA* promintron (from -1 to -86 being +1 the A of the ATG initiation codon of the *rolA* gene). *RolA* coding region indicates the sequence coding for the NH<sub>2</sub> terminal 40 amino acids of the *rolA* protein. GUS indicates the *uidA* coding region. The constructs were cloned in both orientation, as EcoRI fragments, in the vector pG, a derivative of pMB393 (Gage, D.J., Bobo, T. and Long, S.R. (1996) J. Bacteriology, 178, 7159-7166, see materials and methods).

Figure 2. Growth phase dependent expression of  $\beta$ -glucuronidase activity in *Rhizobium leguminosarum*

containing the 86AGUS construct. Cells were grown in rich media (Tryptone-Yeast Extract rich media) until stationary phase (first sample on the negative side of the temporal axis), then diluted one hundred folds in TYR media. The optical density at 600 nm (open squares) and specific  $\beta$ -glucuronidase activity (closed squares) are shown. The values are means of at least four independent experiments. SE means standard error. The construct  $\Delta$  GUS, containing just the *uidA* (GUS) coding region, showed undetectable levels of specific  $\beta$ -glucuronidase activity and consequently has not been represented in the figure.

Figure 3. Root nodules from *Vicia hirsuta* plants infected with *R.l. viciae* containing the 86AGUS construct were stained for  $\beta$ -glucuronidase activity at different age. Panel A to D show one week old nodules. Panel A shows only few layers of cells infected, and consequently (blue)stained, just behind the meristem (indicated with an asterisk in panel E). Panel B shows that in this developmental stage the infection zone is enlarging and will occupy later (Panel C) the whole nodule. Panel D shows three nodules at three different stages of infection on the same root. Panel E shows a two weeks old nodule; note the cylindrical shape of the nodule where all the inner tissue is invaded by bacteroids expressing GUS activity. Panel F shows a three weeks old nitrogen fixing nodule where Zone II and III are (blue) stained while the senescent Zone IV is mostly not stained. Panel G shows a four weeks old nodule displaying an intense staining in the late senescent Zone closest to the root. Panel H shows a double staining for GUS activity and for starch deposition (see Materials and Methods); Note the interzone II-III indicated by the arrows. Above the interzone II-III is visible an invasion Zone II reduced in size and yet still (blue) stained; the double arrow shows (blue) spots of GUS activity closest to the root.

Figure 4. Panels A to E1: roots infected with wild type *R.l. viciae* containing either the 86AGUS construct (in panels C, D and on the right side of panels A and G) or the promintron-*iaaM-tms2* construct (panels B, E, E1, F and on the left side of panels A and G). Panels F and G are microscopic bright field images. Note that the root on the left side of panel A has been infected with bacteria containing the promintron-*iaaM-tms2* construct and has bigger nodules, clustered close to the seed and followed by a long region of the root without any nodule. The most distal part of the root has again nodules. The root on the right side of panel A has been infected with *R.l. viciae* containing the 86AGUS construct: it shows a regular emersion of nodules all along the root (amplified in panel D). Panels B and C allow to compare the size of nodules elicited with wild type (panel C) and genetically modified *Rhizobia* containing the promintron-*iaaM-tms2* construct (panel B). Panel E and E1 show the morphology of two typical nodules elicited by bacteria harbouring the promintron-*iaaM-tms2* construct: bilobate in panel E or highly clustered in panel E1. In panel F is shown a dark field microscopic view of a clarified (see Materials and Methods) bilobate nodule derived from inoculation with *R.l. viciae* containing the promintron-*iaaM-tms2* construct. In panel G the same couple of nodules shown in panel F is compared to a nodule of an identical age derived from a *R.l. viciae* harbouring the 86AGUS construct and stained for  $\beta$ -glucuronidase activity.

Figure 5. *Vicia hirsuta* plants were grown in seed-growth pouches (Mega International, Minneapolis), five seeds per pouch. On the left part of panels A and B, and in panel C are shown 35 days old plants infected with *R.l. viciae* harbouring the promintron-*iaaM-tms2* construct; on the right part of panels A and B, and in panel D are shown plants infected with *R.l. viciae* containing the 86AGUS construct. Note in panels A and B

the bigger size of plants infected with the promintron-*iaaM-tms2* construct in comparison to that one of plants infected with *Rhizobia* harbouring the 86AGUS construct. Plants nodulated by *R.l. viciae* containing the 86AGUS construct have (panel D) smaller leaves, less branches and more senescent leaves per plant. Leaves with a more dark green colour are displayed by plants nodulated by *Rhizobia* harbouring the promintron-*iaaM-tms2* construct (panel C), furthermore the plants do have a more healthy appearance (compare panel C with panel D).

Figure 6. Acetylene reduction assays (ARA). Data compare ARA of 18, 27 and 31 days old plants nodulated either with *R.l. viciae* harbouring the 86AGUS construct (control) or with *Rhizobia* harbouring the promintron-*iaaM-tms2* construct. In each case the control is considered as 100% and data indicate the percentage of increase of ARA obtained from roots nodulated with *Rhizobia* containing the promintron-*iaaM-tms2* construct. Each column represents the medium value obtained from fifty plants. ARA has been expressed in arbitrary units.

Figure 7. Plant dry weight. The stem of nodulated plants was cut just above the seed and dried in an oven at 85°C overnight. Panel A shows the plant dry weight of 18, 27 and 31 days old plants nodulated with *R.l. viciae* harbouring the 86AGUS construct (control) and those infected with *Rhizobia* containing the promintron-*iaaM-tms2* construct. In each case the control is considered as 100% and indicated as zero on the abscissa axe; data indicate the percentage variation, from the corresponding control, of dry weight obtained from stems of plants nodulated with the promintron-*iaaM-tms2* construct. Each column represents the value from 50 plants.

Figure 8. Nucleotide sequence of the *rolA* promintron from Ri plasmid A4 of *Agrobacterium rhizogenes*. Sequences showing homology to: i) the -10 and -35 regions of sigma70-dependent promoters, ii) -10

region of sigma38-dependent promoters, and iii) so-called GEAR-BOX sequence are printed in bold. The arrow indicates the main initiation of transcription.

Figure 9. 29 (panels 1 and 5), 36 (panels 2 and 6),  
5 43 (panels 3 and 7), and 50 (panels 4 and 8) day old root  
nODULES generated on *Vicia hirsuta* plantlets by the wild  
type strain *R.l. viciae* RPR1105 (panels 1-4) or by the  
promintron-iaaM<sub>ms2</sub> construct (panels 5-8). Note the  
increase in nodule size and the different shape of  
10 nodules in 5, 6, 7 and 8 compared to control nodules in  
panels 1, 2, 3 and 4.

Figure 10. Root nodules of *Vicia hirsuta* plants,  
nodulated by the w.t. strain of *R.I. viciae* RPR1105 or by  
its derivative containing the promintron-iaaM-tms2  
15 construct were cut from roots at 14, 21, 28, 35, 42 and  
49 day after infection. Panel B shows a diagram of the  
average weight per plant ( $M_{\text{nod}}(\text{mg})/\text{pp}$ ); standard error is  
indicated. Each point represents the average root nodule  
weight per plant and it results from at least 200  
20 screened nodules. Panel A shows the pendum of obtained  
lines.

Figure 11. Thin sections (toluidine blue stained)  
of five week old root nodules generated by the wild type  
strain *R.l. viciae* RPR1105 (panels 1 and 2), by the  
25 promintron-iaaM<sub>ms2</sub> construct (panels 3 and 4) or by the  
86AGUS construct (panel 5). Panels 2 and 4 are  
magnification of the nodule meristem of the two nodules  
shown in 1 and 3 respectively. The meristematic activity  
is absent in panel 5, is present at the tip of the nodule  
30 in panel 1 and 2, and is extremely active and enlarged on  
both sides in panel 3 and 4, where small dividing cells  
are present on the cap of the nodule with a large zone of  
recently bacterially infected cells (light stained).

Materials and Methods used are herein depicted:

Construction of recombinant plasmids

Standard techniques were used for the construction of recombinant DNA plasmids. The 86AGUS, 85-17GUS and ΔGUS constructs were subcloned in both orientations as EcoRI fragments in the plasmid vector pG, a derivative of pMB393 (Gage, D.J., Bobo, T. and Long, S.R. (1996), ibidem) obtained by deletion of a 750 bp long EcoRI fragment, and then introduced by electroporation into *Rhizobium leguminosarum* biovar *viciae*, strain LPR1105, a rifampicin resistant derivative of RCR1001 (Hooykas P.J.J. et al. (1977) J. Gen. Microbiol., 98, 477-484). The construct pG-promintron-*iaaM-tms2*, comprising the *rolA* promintron driving the *iaaM* and *tms2* genes is described in Example 3.

Growth conditions and GUS assay

*Rhizobium l. b. viciae* cells harbouring the different constructs were grown in TYR medium at 30°C. Growth curves were determined by measuring OD<sub>600</sub>.

Bacteria, collected by centrifugation, were resuspended in 50mM Na-phosphate buffer (pH 7.0), 10mM β-mercaptoethanol, 10mM Na<sub>2</sub>EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, sonicated for 5 min twice and centrifuged at 16.000 × g for 10 min at 4°C. GUS activity was assayed by using the supernatant. Fluorimetric GUS assays were performed as described by Jefferson (Jefferson, R. (1987) Plant Mol. Biol. Report, 5, 387-405). Protein concentration in bacterial extracts was determined using the Bradford reagent (BIORAD) according to the manufacturer's instructions.

*Rhizobia* harbouring the different constructs were plated and selected on TYR rich media supplemented with 100 µg/ml spectinomycin, and replicated on plates containing 100 µg/ml rifampicin. Strains to be inoculated on plants were grown until stationary phase, centrifuged 5' at 12.000g, washed in PBS buffer pH 7.4 and resuspended in the same buffer. Optical Density (OD) was

measured at 600 nm and  $10^5$  cells were applied on each seedling and incubate for 1 hour. *Vicia hirsuta* seeds were surface sterilised in 5% hydrogen peroxide for 30' and then extensively washed with sterile bidistilled water. Seeds were allowed to swollen over-night at 4°C and then germinated on 1.5% agar plates for three days in the dark. After infection, germinated plantlets were grown in seed-germination pouches (MEGA International Minneapolis, USA) containing 10 ml of Jensen media (Vincent, J.M. (1970) I.B.P. Handbook No. 15, Oxford, Blackwell Scientific publications). Plants were grown in 70% humidity, 16/8 light/dark period at 17 or 22°C.

In situ GUS staining

Roots from nodulated plants were cut and incubated at 37°C overnight in X-Gluc Reagent mix (1mM X-Glucuronide, 0.1mM NaPO<sub>4</sub>, 10mM EDTA pH7.0, 0.5mM K ferrocyanide pH 7.0, 0.5mM K ferrocyanide pH 7.0, 0.1% Triton X-100) then fixed in 4% paraformaldehyde in PBS buffer (GUS Protocols by Gallagher S.G., 1992, Academic Press, Inc.). Roots were then dehydrated in increasing concentration of absolute ethanol and clarified by immersion in a mixture 2:1 benzyl benzoate-benzyl alcohol (Sigma). For starch staining whole nodules were immersed for 20 seconds in an aqueous solution of 0.1M potassium iodide. Whole nodules were photographed with a Nikon microscope in bright-field and epipolarization optics.

Acetylene reduction assays (ARA) were performed by cutting nodulated roots (4 roots per 15 ml tube) and injecting 1 ml of acetylene for at least one hour at room temperature. Acetylene and ethylene peaks were obtained by injecting one millilitre from each tube into a Perkin Elmer Sigma 3B gas chromatograph equipped with a column Porapack Q equipped with a Perkin Elmer 561 recorder.

Example 1: The spliceosomal intron of the *rolA* gene from *Agrobacterium rhizogenes* is a promoter active in Rhizobia.

The DNA sequence, hereafter called promintron, spanning the *rolA* intron (which is 85 bp long and it goes from position -1 up to position -86, being +1 the A of the ATG initiation codon of the *rolA* gene) is able to drive prokaryotic expression in *Rhizobia*. A reporter gene construct (86AGUS; Fig.1) was built by fusing the coding region of the *uidA* gene (Jefferson, 1987, ibidem) to a fragment comprising the DNA sequence coding for the NH<sub>2</sub>-terminal 40 amino acids of the RolA protein preceded by the promintron (i.e. 85 bp of the *rolA* promintron (SEQ ID No.1) plus the A immediately preceding the ATG initiation codon of the *rolA* coding region). GUS activity was detected by fluorimetric assays in extracts from *Rhizobium leguminosarum* biovar *viciae* (*R. l. viciae*) harbouring the construct 86AGUS (Fig.2). A similar pattern of expression (data not shown) has been obtained by using the 85-17GUS construct which comprises the promintron of SEQ ID No. 1 and a 17 bp linker (SEQ ID No. 2) and the *uidA* coding region (Fig. 1). The construct ΔGUS consisting of the *uidA* coding region by itself (Fig.1) gave no GUS activity in *Rhizobia* harbouring such construct (data not shown). *R. l. viciae* strain LPR 1105 harbouring either of the three constructs were grown in Tryptic-Yeast Extract (rich media) up to stationary phase at an optical density of 1.8 at 600 nm, then diluted 100 times in the same media. Samples were taken in early, mid and late exponential phase of growth and then in early and late stationary phase of growth. GUS activity was detected only in protein extracts from *R. l. viciae* harbouring the 86AGUS (fig.2) or the 85-17GUS (data not shown) constructs while ΔGUS showed undetectable levels of β-glucuronidase activity, as untransformed *Rhizobia*. The data obtained show that, in *Rhizobia* harbouring the 86AGUS (or 85-17GUS constructs), β-glucuronidase activity declines during the exponential phase of growth and it increases in early stationary phase and it reaches the

highest value at late stationary phase of growth (Fig.2). The same experiment was repeated diluting 100 times the stationary culture grown in rich media into a minimal defined media containing a concentration of mannitol, used as carbon source, limiting for growth. After the dilution, a long period of growth lag was observed followed by an exponential phase of growth and by a stationary phase of growth reached at an optical density of 0.6. This reduced O.D. is caused by the carbon limiting condition applied. Promoters of enteric bacteria regulated by the stationary sigma factor sigma S are specifically activated under these conditions. The promintron sequence harbours a sigma S-like consensus sequence (Fig.8). The GUS activity in *Rhizobia* harbouring the 86AGUS construct was enhanced during the stationary phase of growth and the values reached were 2-times higher than those measured under stationary phase of growth reached in rich media as a consequence of the carbon starvation. Thus, gene expression driven by the promintron sequence is inversely correlated to growth rate and it is induced at the onset of the stationary phase of growth. An identical pattern of expression has been found utilising the 85-17GUS construct where the promintron sequence is separated from the *uidA* gene by a 17 base pairs polylinker shown in SEQ ID No. 2. Thus, the 85 bp long promintron has a promoter function in *Rhizobia*. Open squares represent the O.D. at 600 nm; filled squares represent GUS activity.

Example 2: The promintron drives gene expression within nodules with a novel and unexpected pattern of expression.

*Rhizobium leguminosarum* biovar *viciae* strain LPR 1105 harbouring the 86AGUS construct or the 85-17GUS or the ΔGUS, where used to infect *Vicia hirsuta* plants. The plantlets were grown under greenhouse conditions up to 60 days after planting, and at different times roots were

cut, fixed under buffered paraformaldehyde and stained for in situ GUS activity (Jefferson, 1987, *ibidem*), after over-night incubation in the appropriate buffer, roots were dehydrated by immersing into increasing ethanol  
5 solutions followed by toluene clarification and observed under an optical bright field microscopy. DNA fragments comprising the promintron DNA sequence shown in SEQ ID No. 1 promotes expression in the nodules within symbiotic *Rhizobia* with a characteristic and novel pattern of expression resulting in a constitutive expression of the gene of interest. For constitutive expression it is meant that expression, although timed in a specific pattern, overall it takes place in all the zones of the nodule where plant cells do contain bacteroids. This expression  
10 pattern is strikingly different from that one shown by either sigma 54 or sigma 70-like regulated promoters, for comparison see Sharma S.B. and E.R. Signer (1990) *Gene Dev.*, 4, 344-356.

20 *Rhizobium* bacteria when entering into the host plant are called bacteroids. Bacteroids are known to undergo 5 differentiation forms progressively older from stage 1 to 5. The different stages are described in Vasse, J., et al. (1990) *J. Bacteriol.*, 172, pp. 4295-4306. In an indeterminate nodule such that of *Vicia hirsuta* the youngest bacteroids are located in the early infection Zone II, close to the tip meristem (which represents Zone I) outgrowing from the root, where bacteria are recently released from the infection threads crossing first the root hairs and then the cortical layers to reach finally the growing nodule. Much older bacteroids are located in the late infection Zone II, then in the nitrogen fixing Zone III and finally in the senescent Zone IV only ghost membrane of bacteroids are present. Expression driven by the promintron was observed  
25 in Zones II, III and IV of the nodule at any age including in the very old senescent zone in the region  
30  
35

most proximal to the root (see Figure 3). Thus, all plant cells harbouring bacteroids at any stage do have  $\beta$ -glucuronidase activity within their bacteroids. The pictures are taken from nodules infected by *Rhizobia* containing the 86AGUS construct. An identical pattern of expression has been obtained utilising the 85-17GUS construct that gives only a slightly weaker signal. No staining has been observed with *Rhizobia* harbouring the construct  $\Delta$ GUS. In Fig. 3, panel I, is presented a double staining for GUS (blue) activity and for starch (dark brown). In older nodules starch deposition occurs in Zones III and IV, but it is mostly used because its staining describes the interzone II-III (see arrow in Fig.3, panel I). The GUS staining is both above and below the interzone II-III.

Example 3: Construction of plasmid pG-promintrone-iaaM-tms2

The recombinant plasmid pG-promintrone-iaaM-tms2 was obtained by ligating the EcoRI-KpnI fragment comprising SEQ ID No. 1 and SEQ ID No. 2, spanning the *rolA* promoter sequence, within the pG plasmid (see materials and methods) cut with EcoRI-KpnI. Afterwards the plasmid pG-promintrone was cut with KpnI-HindIII and the *iaaM* and *tms2* coding sequences were introduced as KpnI-SphI and SphI-HindIII fragments, respectively. The KpnI-SphI fragment of 1775 bp (2+53+1671+49) spans the coding region of the *iaaM* gene from *Pseudomonas syringae* subsp. *savastanoi*. The *iaaM* sequence has been characterised by Yamada et al (1985, ibidem) and the sequence used contains 1773 bp (53+1671+49) from the DraI site located 53 bp before the ATG initiation codon till the SphI site 46 bp after the TAA stop codon. The SphI-HindIII fragment of 1452 (22+13+1403+14) bp spanning the *tms2* coding region from *Agrobacterium tumefaciens* pTi A6 (Klee, H. et al. (1984) P.N.A.S. USA 81, pp. 1728-1732) contains 1403 bp of coding region preceded by 14 bp and followed by 5

bp belonging to the untranslated regions of the *tms2* gene.

Consequently, the plasmid pG-promintron-*iaaM-tms2* obtained possesses the following structural features:

- 5        - a promintron fragment comprising the 85 bp of the *rolA* promintron (SEQ ID No. 1), plus 17 bp of linker sequence (SEQ ID No. 2) and having an Eco RI adapter (sequence GAATTC) at its 5' end;
- 10      - 6 bp as KpnI site (sequence GGTACC) plus extra 2 bases added as linker;
- 15      - 53 bp of 5' untranslated sequence of the *iaaM* gene from *Pseudomonas syringae* subsp. *savastanoi*;
- 20      - 1671 bp of coding region of the *iaaM* gene from *Pseudomonas syringae* subsp. *savastanoi*;
- 25      - 49 bp (46+3 bp of stop codon) of 3' untranslated trailer sequence of the *iaaM* gene from *Pseudomonas syringae* subsp *savastanoi* ending with the SpHI site;
- 30      - 22 bp consisting of a polilinker sequence (CTGCAGGTCGACTCTAGAGGAT, SEQ ID No. 3);
- 35      - 13 bp of untranslated leader region of the *tms2* gene (CCAACTCAGAGAG, SEQ ID No. 4);
- 40      - 1403 bp spanning the coding region of the *tms2* gene from *Agrobacterium tumefaciens* pTiA6;
- 45      - 14 bp at the 3' end including an HindIII site (sequence TAAACATCAAAGCTT, SEQ ID No. 5) being TAA the stop codon, AC sequence at the 3' of the *tms2* coding region after the stop codon, and the rest being HindIII linker sequence (HindIII site being AAGCTT).

Example 4: The promintron-*iaaM-tms2* construct alters root nodule number, location and size.

Vicia hirsuta plantlets were harvested at 18 days after infection with either the 86AGUS construct or the promintron-*iaaM-tms2* construct. In the case of plants infected with Rhizobia harbouring the 86AGUS construct, an average number of 22 nodules per plant was observed while this number decreased at 7.5 nodules per plant in

the case of plants infected with *Rhizobia* harbouring the promintrron-*iaaM-tms2* construct. Considering the control 86AGUS infected plants as 100%, the presence of the bicistronic operon in *Rhizobia* reduces the nodule number 5 by 65%. In contrast to this feature, the nodule size is strongly increased in plants infected with *Rhizobia* harbouring the promintrron-*iaaM-tms2* construct. In Figure 10 4, panels B and C, the size of the nodules can be compared. Not only *Rhizobia* containing the promintrron-*iaaM-tms2* construct induced nodules which were bigger in size, but sometimes the nodules were also bilobate (panel E). In addition, the root nodules elicited by *Rhizobia* harbouring the promintrron-*iaaM-tms2* construct, are 15 localised mostly on the primary root (Fig.4, panel A left) and rarely on secondary roots as is the case with plants inoculated with *Rhizobia* harbouring either the 86AGUS construct or not harbouring any construct at all (Fig.4, panel A, right). Finally, Fig. 4 (panel A, left) shows that nodules elicited by *Rhizobia* harbouring the 20 promintrron-*iaaM-tms2* construct are concentrated in the root region close to the seed, followed by a large zone of the root where no nodule did occur. This was not the pattern of nodulation elicited by wild type *Rhizobium*, which consisted of small nodules distributed uniformly 25 all along the root (Fig.4, panel A right, and panel D).

Example 5: The root nodules induced by *Rhizobia* harbouring the promintrron-*iaaM-tms2* construct shows an increased acetylene reduction activity (ARA)

Nodulated roots infected with wild type *Rhizobium leguminosarum* biovar *viciae* strain LPR1105 or the same 30 strain harbouring either the 86AGUS or the promintrron-*iaaM-tms2* construct were followed for one month after infection and assayed for the ability to reduce acetylene to ethylene. This is the most commonly used indirect 35 enzymatic assay to evaluate the ability of the nitrogenase complex to reduce atmospheric nitrogen to

ammonia. Eight independent experiments using up to fifty plants per point have been carried out at two different temperatures ( $17^{\circ}\text{C}$  and  $22^{\circ}\text{C}$ ). Results show that the promintrон-*iaaM-tms2* construct induces the formation (at  $22^{\circ}\text{C}$ ) of root nodules able to reduce acetylene to ethylene more efficiently than the control root nodules generated either by the wild type *Rhizobia* or by *Rhizobia* harbouring either the 86AGUS or the 85-17GUS. Thus, the presence of the promintrон-*iaaM-tms2* construct increases the capacity of nitrogen fixation of root nodules. Samples were taken at 18, 27 and 31 days after inoculation of germinated seeds with bacteria. Acetylene reduction activity (ARA) was followed at the Gas Cromatograph (see Materials and Methods) and expressed in arbitrary units. In Fig. 6 the media of the value of the control (at least 50 plants in each case) are taken as 100%. Each column indicate the percentage of increase compared to the controls of ARA from roots nodulated with the promintrон-*iaaM-tms2* construct. In function of time, the percentage of ARA increases from 30%, at 18 days, up to 240% more than controls at 31 days.

#### Example 6: Plant growth and biomass

As shown in Fig. 5, 35 days old *Vicia hirsuta* plants nodulated by *Rhizobia* containing the promintrон-*iaaM-tms2* construct are bigger in size (more than 20% increase). Furthermore, these plants are not only bigger, but also altered in their phenotype having dark green leaves and a better vigour in comparison to sister plants inoculated with the wild type *R.l. viciae*. In Figure 7 are listed the dry weights of plants 18, 27 and 31 days old. The dry weight of control plants is considered 100% at any age and indicated as zero. It could be observed a significant decrease (i.e. 24%) in the average dry weight of plants nodulated with *Rhizobia* containing the promintrон-*iaaM-tms2* construct at 18 days, when the influence on plant growth of seed storage proteins is

still important. In contrast, 27 and 31 days old plants, when the nitrogen contained in the seeds is mostly exhausted, shows a 35% and 44%, respectively, increase in dry weight of plants nodulated with *Rhizobia* harbouring the *promintrona-iaaM-tms2* construct in comparison to the dry weight of plants nodulated by the wild type strain of *Rhizobia* or of *R.l. viciae* containing the 86AGUs or the 85-17GUS constructs.

Example 7: Measurements of acetylene reduction

Acetylene reduction assays were performed to compare root nodules generated on *Vicia hirsuta* by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105 or from its derivative containing the *promintrona-iaaMtms2* construct. Up to 23 days after infection the two strains reduce the same amount of acetylene at a similar rate. Five weeks after infection the meristematic activity is arrested in control nodules while is still generating newly infected cells after 8 weeks in nodules inoculated with bacteria containing the *promintrona-iaaMtms2* construct. At 39 days after infection the acetylene reduction reach a 100% increase when the *promintrona-iaaMtms2* construct is compared to the wild type strain.

Example 8: Increase of stem dry weight

An analysis of *Vicia hirsuta* stem dry weight was performed to compare *Vicia hirsuta* plants nodulated by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105 with its derivative containing the *promintrona-iaaMtms2* construct. The plant biomass was substantially unaffected up to the 23 day after infection. However a 15% increase at 51 days after infection was observed when the *promintrona-iaaMtms2* construct is compared to the wild type strain.

Example 9: Total nitrogen content analysis

The analysis of *Vicia hirsuta* stem dry weight was followed by mineralized total nitrogen content analysis.

The total nitrogen content, as elementary N, was measured according to the method of Dumas (Simonne, et al., J. Sci. Food Agric. 73, 39-45) using a Nitrogen Analyzer Macro-N (Foss Heraeus Analysensysteme, Hanau Germany).

5 The total nitrogen concentration is unaffected up to 51 days after infection on nodulated *Vicia hirsuta* plants both with the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105, and with its derivative containing the *promintron-iaaMtms2* construct.

10 Example 10: Analysis of the root nodule weight

An analysis of the root nodule weight was performed to compare plants nodulated by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105, with its derivative containing the *promintron-iaaMtms2* construct. Figure 9 shows 29, 36, 43 and 50 day old root nodules (panels 1,5; 2,6; 3,7; 4,8; respectively) generated on *Vicia hirsuta* plantlets by the wild type strain *R.l. viciae* 1004 (panels 1-4) or by the *promintron-iaaMtms2* construct (panels 5-8). The total mass of nodules is unaffected up to four week after infection with both strains. It reaches a two fold increase 42 days after infection when the *promintron-iaaMtms2* construct is compared to the wild type strain. Figure 10B shows a diagram to compare data of nodule weight up to day 49 after infection among the two strains. The panel of Figure 10A shows that the slope of the interpolating line is 0.26 for the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105, whereas a slope value of 0.46 is obtained when the *promintron-iaaMtms2* construct was used.

30 Example 11: Analysis of meristematic activity

An analysis of thin sections stained with 0,02% toluidine blue of five week old root nodules was performed to compare plants nodulated by the *Rhizobium leguminosarum* biovar *viciae* wild type strain RPR1105 with its derivative containing the *promintron-iaaM-tms2*

construct or the 86AGUS construct. Figure 11 shows that the meristematic activity and newly infected cells are absent in nodules generated by the 86AGUS containing bacteria. However they are present but restricted to the very tip of the root when the wild type strain RPR1105 was used; whilst meristematic activity is still very active with many dividing (small) cells present at the cap of the nodule, with many new infected (light stained) cells when the *promintron-iaaMtms2* construct was used. Therefore it is evident that, at the same age, both the size and the shape of generated nodules in the three samples are statistically different.

Example 12: Measurements of acetylene reduction in French bean

Acetylene reduction assays were performed to compare French bean plants nodulated by the wild type strain CE3 of *Rhizobium etli* or by its derivative containing the *promintron-iaaM-tms2* construct. In contrast to the *Vicia hirsuta* indeterminate type of nodule, French bean produces a determinate type of nodule, namely a nodule missing a persistent meristem, similar to those generated on soybean roots. At 23 days after infection the acetylene reduction rates are identical irrespective to the strain utilised for root infection. At 43 days after infection French bean plants inoculated with bacteria containing the *promintron-iaaMtms2* construct show a 100% increase when compared to the wild type *R. etli* CE3.

Example 13: Increase of stem dry weight in French bean

French bean stem dry weight analysis was performed to compare plants nodulated by the wild type strain CE3 of *Rhizobium etli* or by its derivative containing the *promintron-iaaMtms2* construct. The stem dry weight is identical up to day 23 after the infection irrespective to the strain utilised, while at day 48 the stem dry

weight of plants nodulated by the CE3 strain containing the *promintron-iaaMtms2* construct is increased by 15% when compared to 48 days old French bean plants infected with the wild type strain of *R. etli* CE3.

5           Example 14: Analysis of the root nodule weight in French bean

French bean nodule weight analysis was performed to compare plants nodulated by the wild type strain CE3 of *Rhizobium etli* or by its derivative containing the 10 *promintron-iaaMtms2* construct. The nodule weight is identical up to day 23 after the infection, irrespective to the strain utilised, while at day 48 the nodule mass of plants nodulated by the CE3 strain containing the 15 *promintron-iaaMtms2* construct is increased by 50% when compared to 48 days old French bean plants infected with the wild type strain of *R. etli* CE3.

## CLAIMS

1. Use of the promintron sequence of the *rolA* gene from *Agrobacterium rhizogenes* as in SEQ ID NO. 1, or of DNA sequences comprising said promintron sequence, or of functional homologous or portion thereof, to induce the expression of a DNA coding sequence, in recombinant bacteria during exponential, post-exponential and stationary phase of growth, and in bacteroids within root nodules, said coding DNA sequence being under the control of said promintron sequence.
2. Use of the promintron sequence according to claim 1 wherein said recombinant bacteria belong to either the *Enterobacteriaceae* or the *Rhizobiaceae* families.
3. Use of the promintron sequence according to claim 2 wherein said recombinant bacteria belonging to either the *Enterobacteriaceae* or the *Rhizobiaceae* families are *E. coli*, *Rhizobia* or *Agrobacteria*.
4. Use of the promintron sequence according to claim 3 wherein said recombinant bacteria are of the *Rhizobia* genus, either within symbiotic root nodules or in a free living status.
5. Use of the promintron sequence according to claim 4 wherein said recombinant bacteria of the *Rhizobia* genus within symbiotic root nodule, are either bacteroids of stage I, II, III, IV, V, or *Rhizobia* present in the apoplastic space, or *Rhizobia* present in the senescence zone, or *Rhizobia* present in the nitrogen fixing zone, or *Rhizobia* present in the invasion zone.
6. A recombinant DNA molecule comprising the promintron sequence according to claim 1, or functional homologous or portion thereof, and covalently linked to the 3' end of said promintron sequence, a DNA coding sequence, said recombinant DNA molecule being

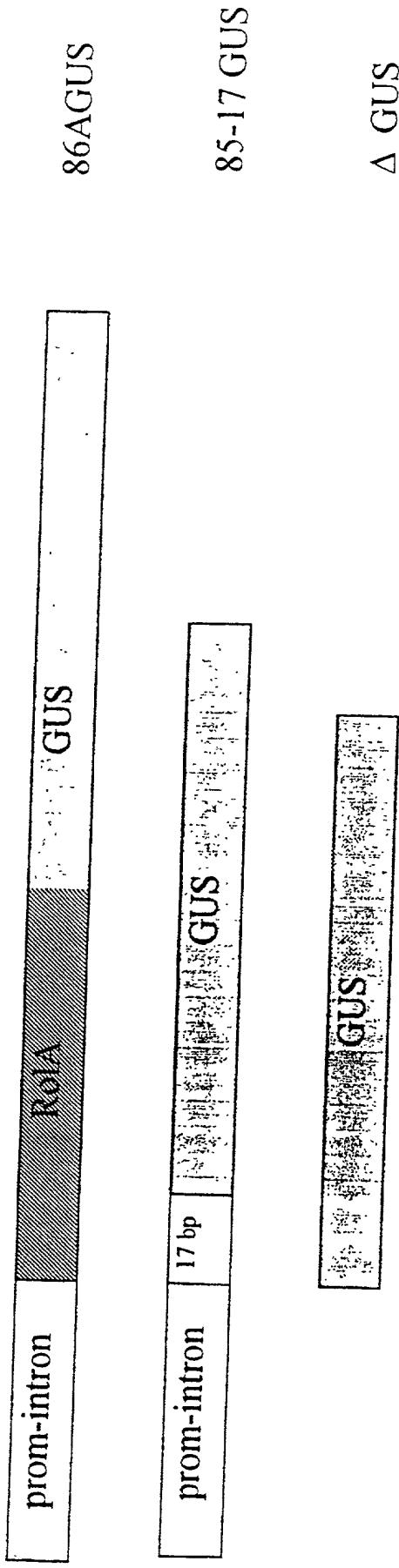
- either harboured by prokaryotic episomal elements,  
or integrated in a bacterial genome.
7. The recombinant DNA molecule according to claim 6  
wherein said DNA coding sequence is either a  
5 monocistronic or a polycistronic transcriptional  
unit.
8. The recombinant DNA molecule according to claim 7  
wherein said DNA coding sequence encodes a protein  
involved in plant hormone auxin synthesis and/or  
10 metabolism.
9. The recombinant DNA molecule according to claim 8  
wherein said DNA coding sequence encodes a protein  
involved in the synthesis and/or metabolism of the  
auxin IAA or of the auxin indolethanol.
- 15 10. The recombinant DNA molecule according to claim 8  
wherein said DNA coding sequence encodes the *iaaM*  
protein from *P. syringae* subsp. *savastanoi* or an  
homologous thereof.
11. The recombinant DNA molecule according to claim 8  
20 wherein said DNA coding sequence encodes the *tms2*  
protein from *A. tumefaciens* or an homologous  
thereof.
12. The recombinant DNA molecule according to claim 8  
25 wherein said DNA coding sequence encodes both the  
*iaaM* and the *tms2* coding regions of claim 10 and 11,  
respectively.
13. The recombinant DNA molecule according to claim 8  
wherein said DNA coding sequence encodes the  
indolepyruvate decarboxylase from *Enterobacter*  
30 *cloacae* or an homologous thereof.

14. Genetically engineered bacteria comprising the recombinant DNA molecule according to claims from 6 to 13.
- 5 15. Use of the recombinant DNA molecule according to claims from 6 to 13 to significantly increase the size of nodules of a plant.
- 10 16. Use of the recombinant DNA molecule according to claim 15 wherein said statistically significant increase of the nodule size is of at least 20%.
- 15 17. Use of the recombinant DNA molecule according to claims from 6 to 13 to significantly increase the capacity to fix nitrogen of a nodulated plant.
18. Use of the recombinant DNA molecule according to claim 17 wherein said statistically significant increase of the capacity to fix nitrogen is of at least 20%.
- 20 19. Use of the recombinant DNA molecule according to claims from 6 to 13 to significantly increase the plant biomass production.
- 25 20. Use of the recombinant DNA molecule according to claim 19 wherein said statistically significant increase of the plant biomass production is of at least 10%.
21. Legume plant infected by bacteria harbouring the recombinant DNA molecule according to claims from 6 to 13 and having a significant increase of the size of nodules, and/or of the nodule capacity to fix nitrogen, and/or of the plant biomass, and/or of the ability to fix nitrogen.

PROMOTER REGION

REPORTER GENE

CONSTRUCTS



1/12

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FIG. 1

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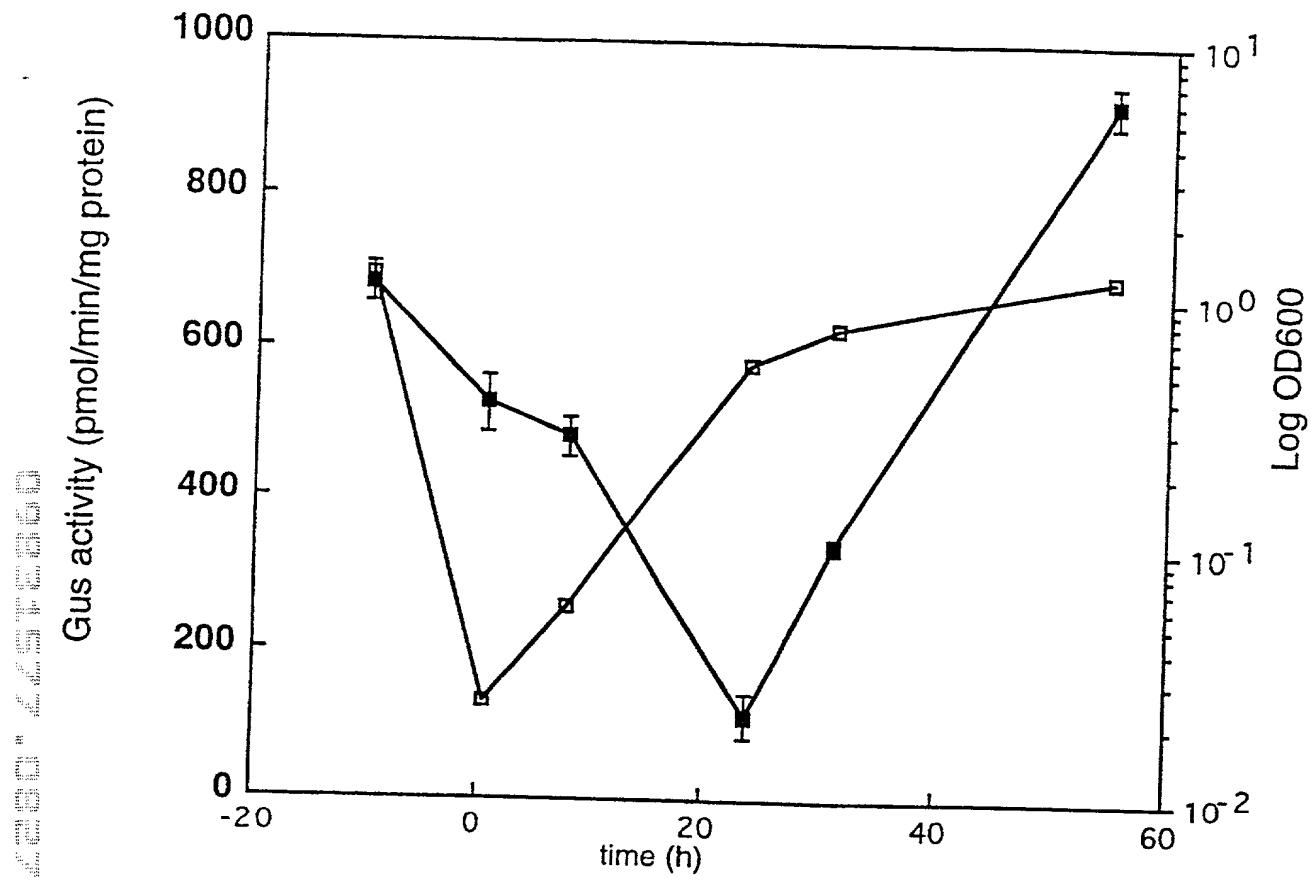


FIG. 2

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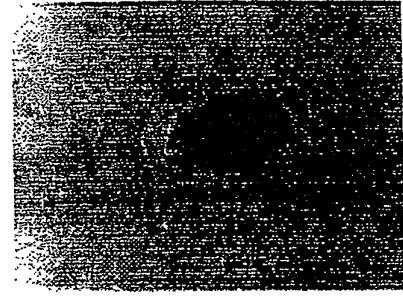
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A



B



C



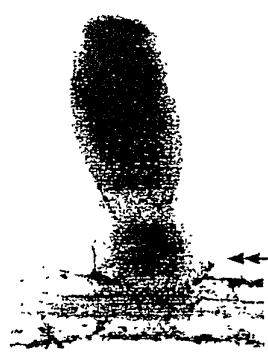
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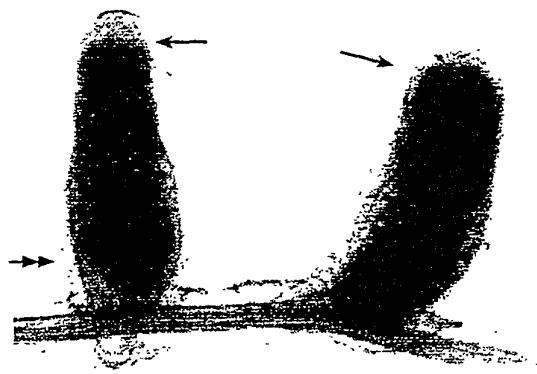
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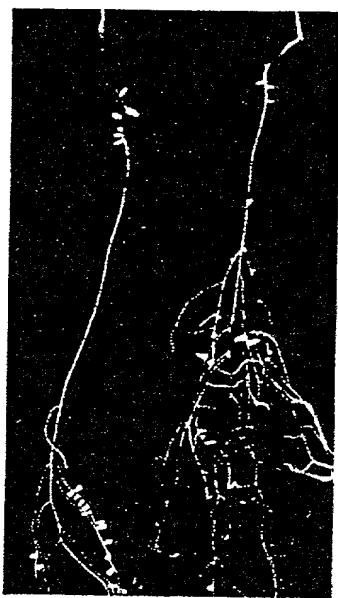
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FIG. 3

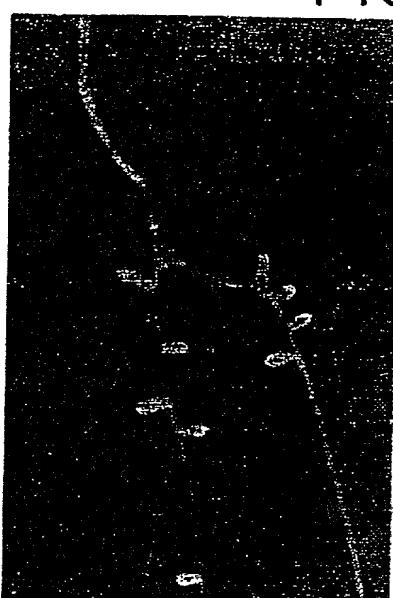
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FIG. 4



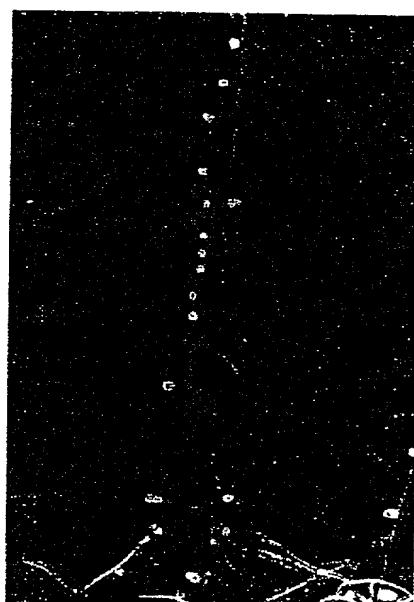
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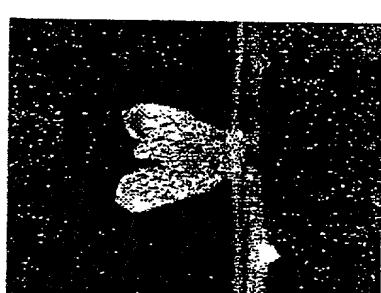
B



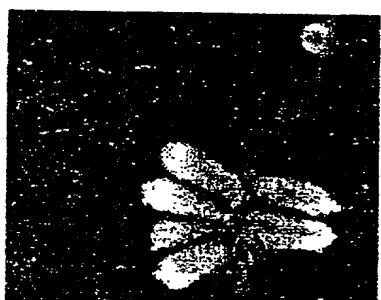
C



D



E1



E



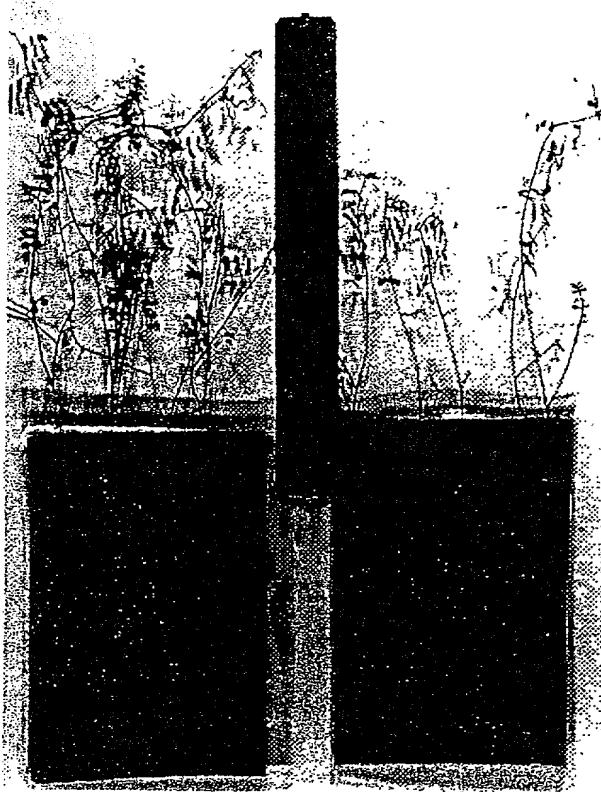
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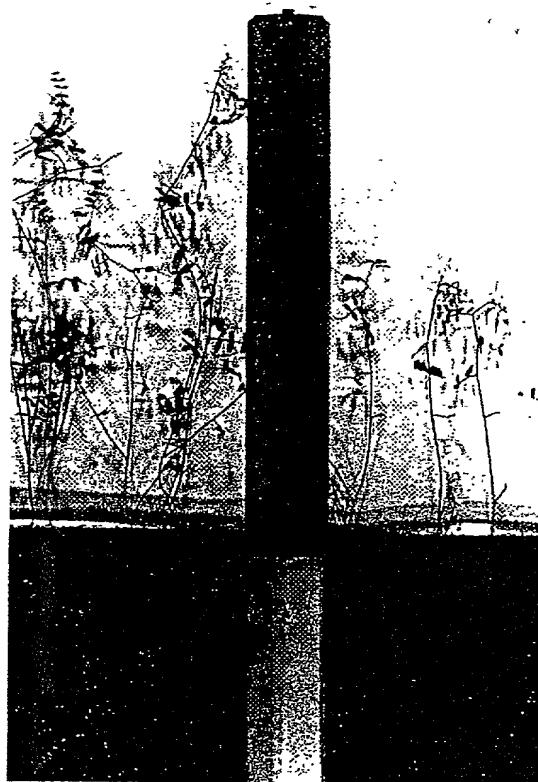
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09/831577

5/12



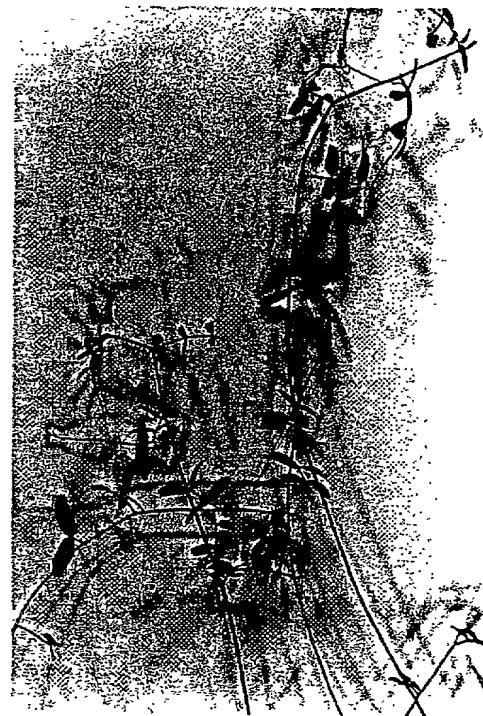
**A**



**B**



**C**



**D**

**FIG. 5**

09/831577

6/12

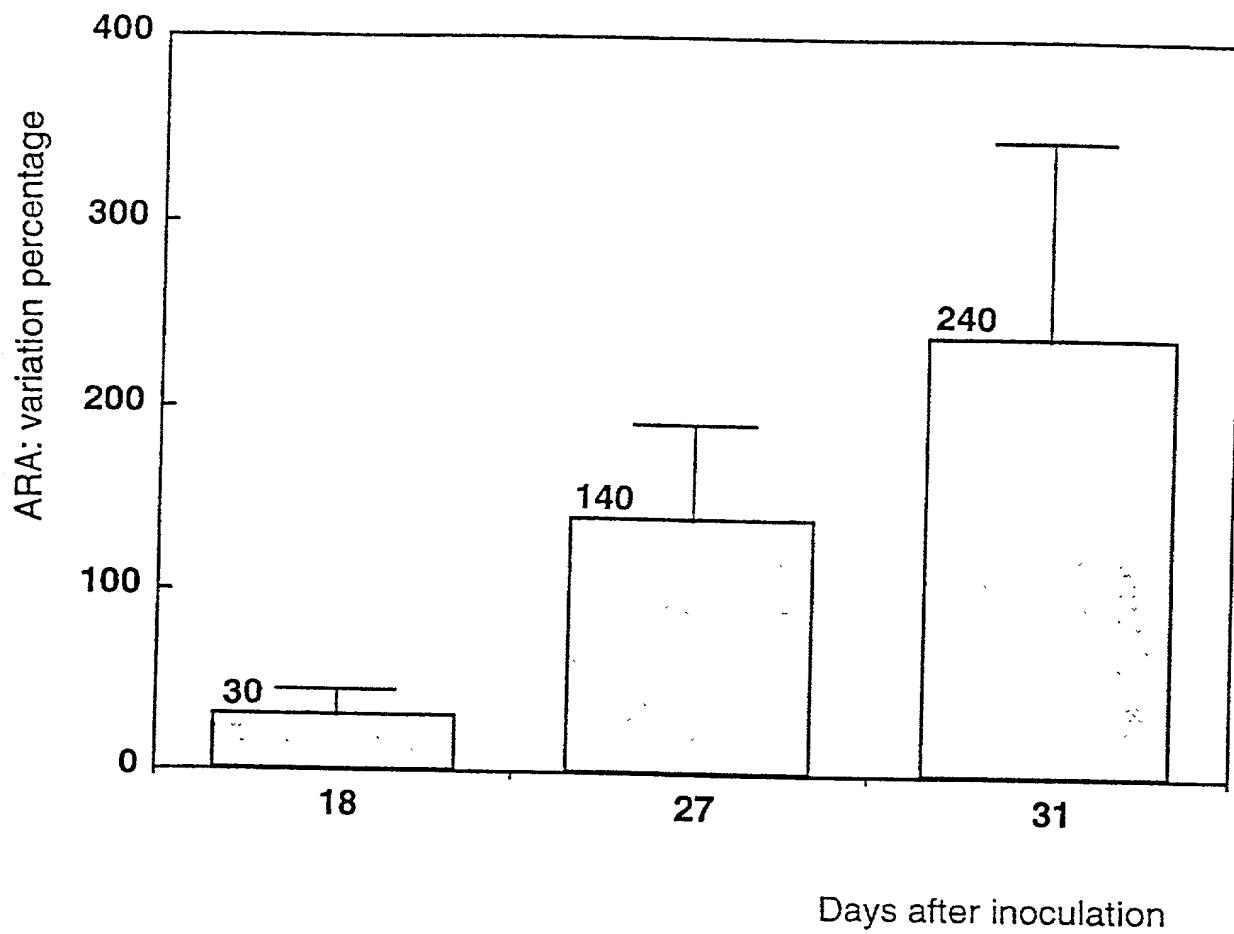


FIG. 6

09/831577

7/12

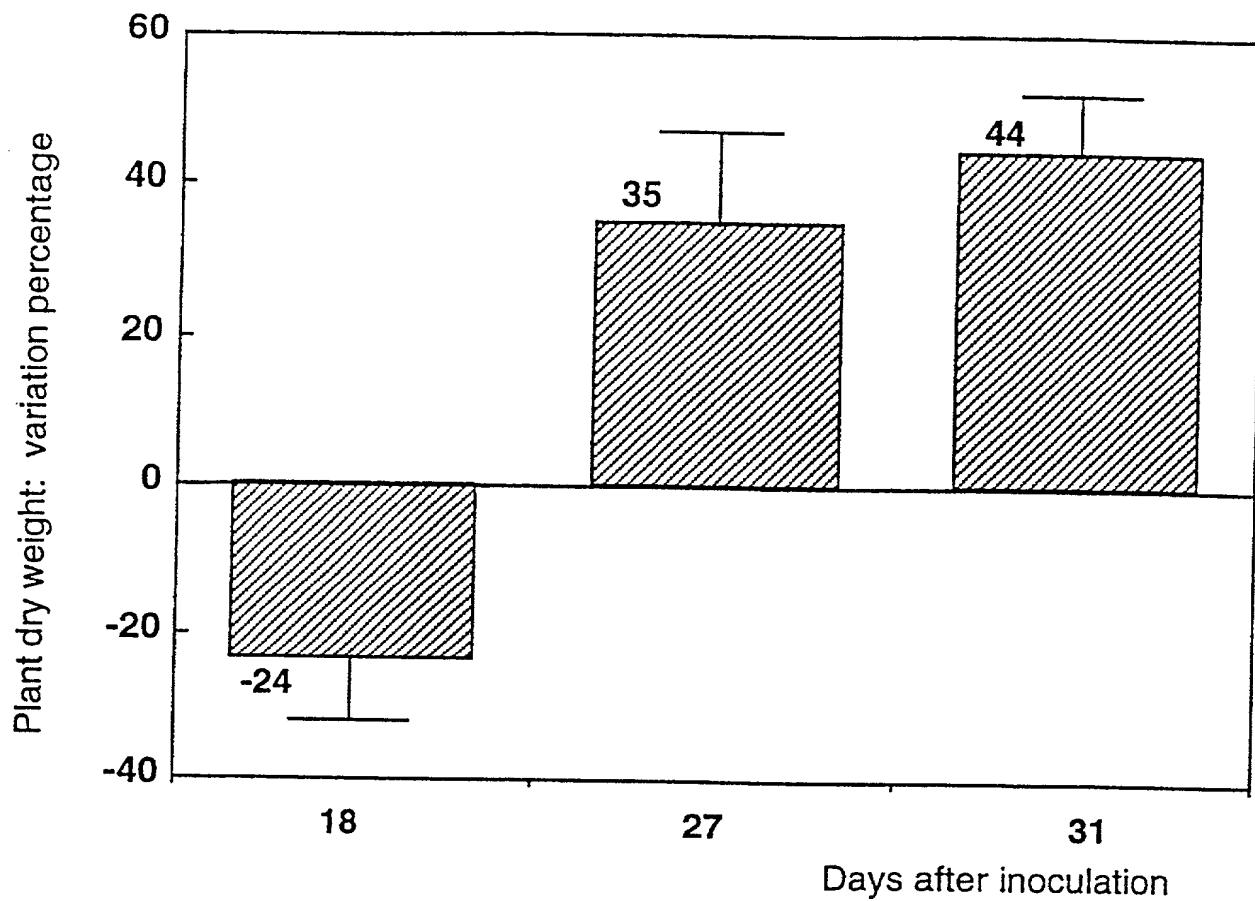


FIG. 7

09/831577

8/12

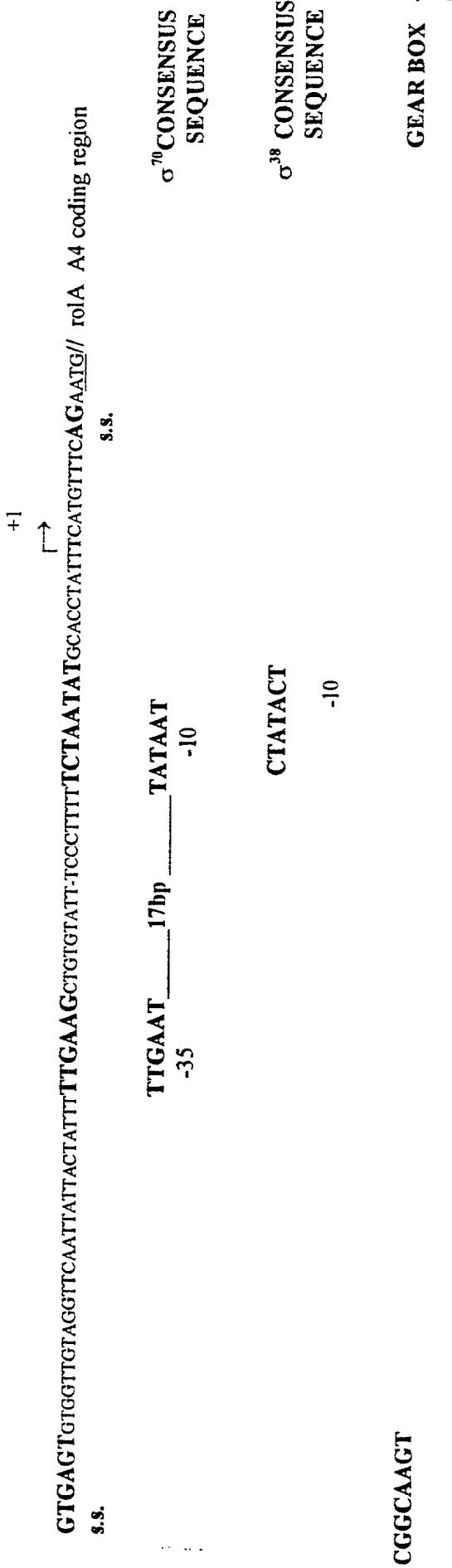


FIG. 8

09/831577

9/12

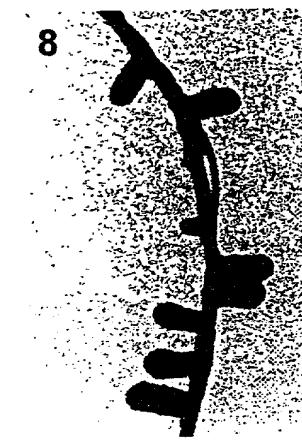
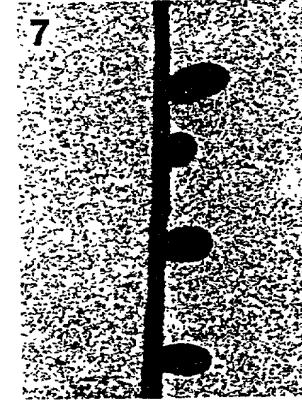
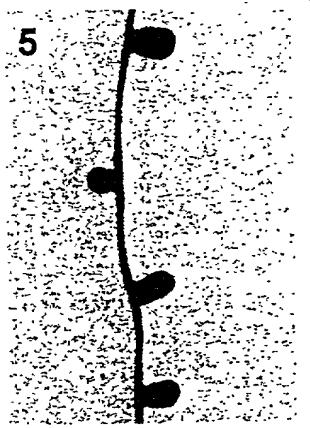
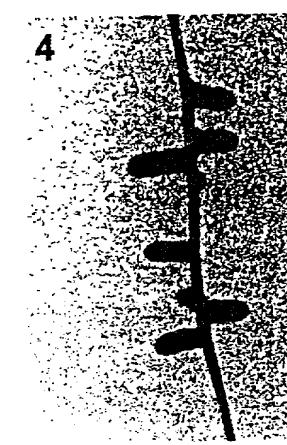
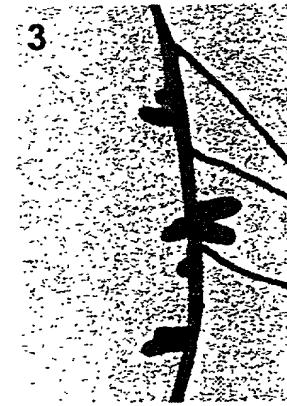
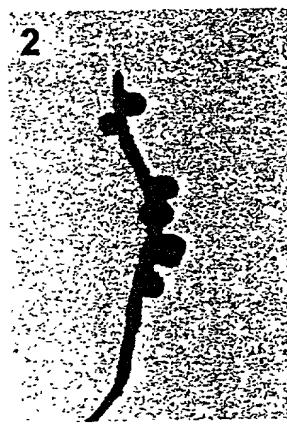
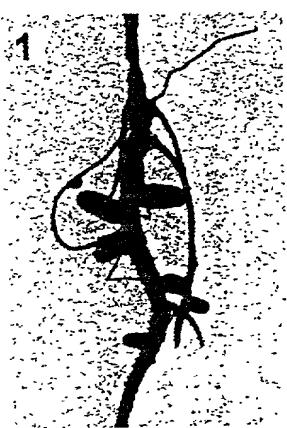


FIG. 9

09/831577

10/12

Days A. I.	Mnod(mg)/pp	
	RPR 1105	<i>iaaMtms2</i>
14	4 ± 1	5,0 ± 0,4
21	5 ± 1	7 ± 2
28	8 ± 2	12 ± 3
35	11 ± 1	12,2 ± 0,3
42	11 ± 4	23 ± 6
49	13 ± 3	18 ± 4

pendence	RPR 1105	0,26700	±	3.E-05
intercept	RPR 1105	0,26	±	0,03

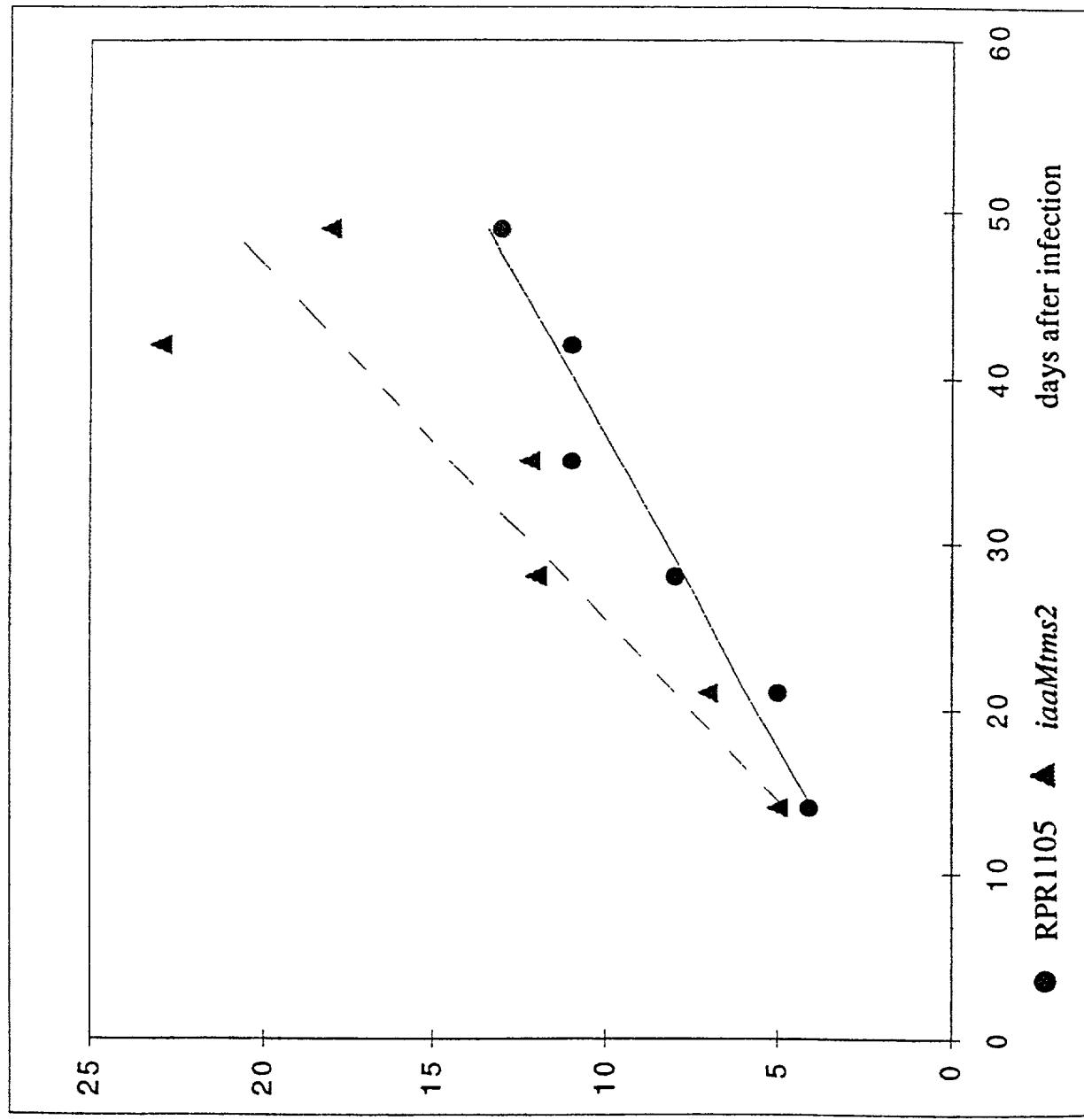
pendence	<i>iaaMtms2</i>	0,4620	±	1.E-04
intercept	<i>iaaMtms2</i>	-1,69	±	0,1

FIG. 10A

09/831577

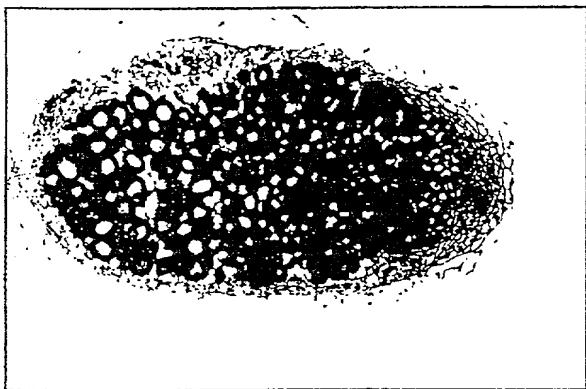
11/12

FIG. 10B

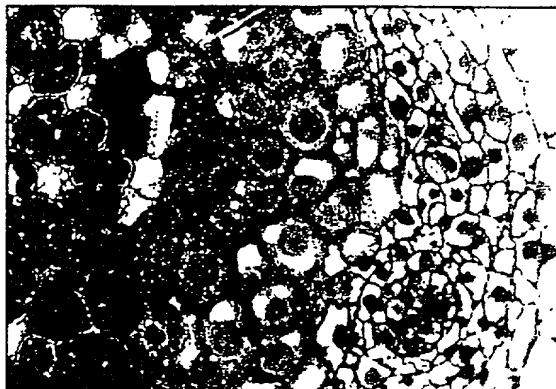


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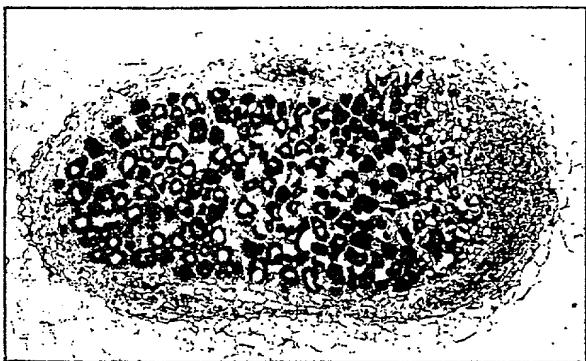
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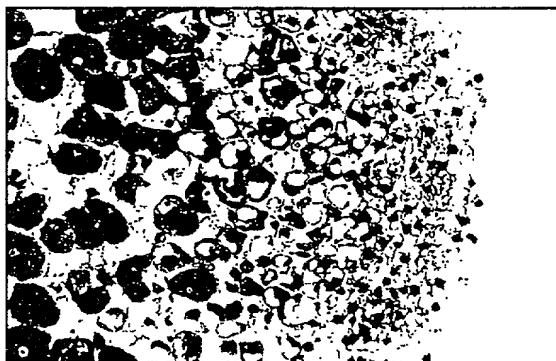
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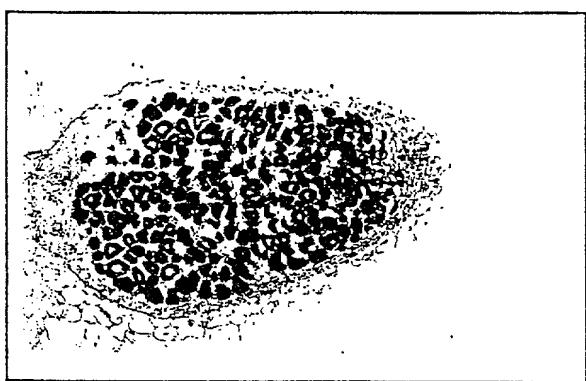
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3



4



5

FIG. 11

# DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

## MODULO DI DICHIARAZIONE PER DOMANDA DI BREVETTO

### ITALIAN LANGUAGE DECLARATION

Io, sottoscritto inventore, dichiaro con il presente che:

Il mio domicilio, recapito postale e cittadinanza sono quelli indicati in calce accanto al mio nome.

Che mi reputo in buona fede essere l'inventore originario, primo e unico (qualora un solo nominativo appaia elencato appresso) o il coinventore (qualora i nominativi siano più di uno) primo e originario dell'invenzione da me rivendicata, e per la quale faccio domanda di brevetto. Tale invenzione è chiamata:

METHOD TO CONTROL GENE EXPRESSION IN BACTERIA  
NAMELY RHIZOBIAEAE, TO IMPROVE ROOT NODULE DEVELOPMENT..

E la sua descrizione è allegata alla presente Dichiarazione a meno che non sia spuntata la seguente casella:

(...) il  
è stata depositata una domanda di brevetto  
statunitense numero o una domanda di brevetto  
internazionale PCT numero  
che è stata modificata il  
(se del caso)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

the specification of which is attached hereto unless the following box is checked:

(x) was filed on NOVEMBER 8, 1999  
as United States Application Number  
or PCT International Application Number  
PCT/IT99/00355  
and was amended on  
(if applicable)

Dichiaro inoltre con il presente di aver letto e compreso il contenuto della descrizione sopra indicata, comprese le rivendicazioni, come rettificata da qualsiasi emendamento a cui si sia accennato sopra.

Riconosco il mio dovere di rivelare informazioni che costituiscono materiale per l'esame della presente domanda secondo i termini del Titolo 37, Codice dei Regolamenti Federali, Comma 1,56(a).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1,56(a).

## Italian Language Declaration

Con il presente rivendico i benefici di priorità per l'estero come stabilito dal Titolo 35, Codice degli Stati Uniti, Comma 119 per qualsiasi domanda di brevetto (o brevetti) straniera o per qualsiasi certificato di invenzione sotto elencato, ed ho anche elencato qui sotto tutte le domande di brevetto e certificati d'invenzione stranieri aventi una data di presentazione anteriore a quella della domanda per la quale si rivendica la precedenza:

Prior foreign applications  
Domande all'estero precedenti

Priority claimed  
Priorità rivendicata

(Number) (Numero)	(Country) (Paese)	(Day, Month, Year Filed) (Giorno, Mese, Anno di Deposito)	(X)	(...)	(...)	(...)	(...)
SE 98830674.2	EPO	9 - NOVEMBER - 1998	(X)	Yes	No	Yes	No
(Number) (Numero)	(Country) (Paese)	(Day, Month, Year Filed) (Giorno, Mese, Anno di Deposito)	(...)	(...)	(...)	(...)	(...)
(Number) (Numero)	(Country) (Paese)	(Day, Month, Year Filed) (Giorno, Mese, Anno di Deposito)	(...)	(...)	(...)	(...)	(...)

Con il presente rivendico il beneficio previsto dal Titolo 35, Codice degli Stati Uniti, Comma 120, per qualsiasi domanda (o domande) di brevetto sotto indicate, ed entro i limiti nei quali il materiale indicato in ciascuna delle domande di brevetto non è stato rivelato nella precedente domanda di brevetto americana nel modo previsto dal primo paragrafo del titolo 35, Codice degli Stati Uniti, Comma 112, riconosco il mio dovere di rivelare il materiale d'informazione, così come viene definito nel titolo 37, Codice dei Regolamenti Federali, Comma 1,56(a), che possa essere venuto ad aggiungersi nel periodo intercorso tra la data di presentazione della domanda precedente e la data nazionale o internazionale da PCT di presentazione di questa domanda:

(Application Serial No.) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, pendente, abbandonata)	(Legal Status) (patented, abandoned)	pending,
(Application Serial No.) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, pendente, abbandonata)	(Legal Status) (patented, abandoned)	pending,

Dichiaro inoltre con il presente che tutte le informazioni da me fornite sono per quanto mi consta vere e che tutte le affermazioni da me fatte sono per quanto mi consta vere; dichiaro inoltre che quando ho fatto queste affermazioni ero al corrente del fatto che false dichiarazioni fatte intenzionalmente sono punibili con multa o incarcerazione o ambedue, secondo quanto stabilito dalla sezione 1001 del Titolo 18 del Codice degli Stati Uniti e che tali informazioni intenzionalmente false possono mettere a repentaglio la validità della domanda di brevetto rilasciata in base ad esse.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

## Italian Language Declaration

PROCURA: Io, sottoscritto inventore, nomino con la presente il seguente Procuratore (o Procuratori) o Agente (o Agenti) che si incarica di perseguire questa pratica e di portare a termine tutte le operazioni necessarie all'Ufficio Brevetti pertinenti a questa pratica. (Elencare il Nome e il Numero di Matricola)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

Recapito per la corrispondenza:

Send correspondence to:

SAMVELS, GAUTHIER & STEVENS LLP  
225 Franklin Street, Suite 3300  
Boston, Massachusetts 02110

Telefonare a: (Nome e Numero)

Direct telephone calls to: (name and telephone number):

Tel.: (617) 426-9180  
 Fax.: (617) 426-2275

Nome completo dell'inventore primo o unico	Full name of sole or first inventor	
<u>ROBERTO DE FEZ</u>	<u>Inventor's signature</u>	Date <u>31 MAY 2001</u>
Firma dell'inventore	Data	
<u>R. De Fez</u>		
Residenza	Residence	
<u>VIA MARCONI, 10 - 80125 NAPOLI</u>		
Cittadinanza	Citizenship	
<u>ITALIAN</u>		
Recapito o Casella Postale	Post Office Address	
<u>VIA MARCONI, 10 - 80125 NAPOLI</u>		
Nome completo del secondo inventore, se esistente	Full name of second joint inventor, if applicable	
Firma dell'inventore	Data:	Inventor's signature
<u></u>		
Residenza	Residence	
Cittadinanza	Citizenship	
Recapito o Casella Postale	Post Office Address	
Nome completo del terzo inventore, se esistente	Full name of third joint inventor, if applicable	
Firma dell'inventore	Data:	Inventor's signature
<u></u>		
Residenza	Residence	
Cittadinanza	Citizenship	
Recapito o Casella Postale	Post Office Address	

(Si prega di fornire le stesse informazioni e firme di eventuali terzi e più coinventori)

(Supply similar information and signature for third and subsequent joint inventors)

# **DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

## ***MODULO DI DICHIARAZIONE PER DOMANDA DI BREVETTO***

### **ITALIAN LANGUAGE DECLARATION**

Io, sottoscritto inventore, dichiaro con il presente che:

Il mio domicilio, recapito postale e cittadinanza sono quelli indicati in calce accanto al mio nome.

Che mi reputo in buona fede essere l'inventore originario, primo e unico (qualora un solo nominativo appaia elencato appresso) o il coinventore (qualora i nominativi siano più di uno) primo e originario dell'invenzione da me rivendicata, e per la quale faccio domanda di brevetto. Tale invenzione è chiamata:

**METHOD TO CONTROL GENE EXPRESSION IN BACTERIA  
NAMELY RHIZOBIAEAE, TO IMPROVE ROOT NODULE DEVELOPMENT...**

E la sua descrizione è allegata alla presente Dichiarazione a meno che non sia spuntata la seguente casella:

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è stata depositata una domanda di brevetto  
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internazionale PCT numero  
che è stata modificata il  
(se del caso)

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**METHOD TO CONTROL GENE EXPRESSION IN BACTERIA**

**NAMELY RHIZOBIAEAE, TO IMPROVE ROOT NODULE DEVELOPMENT...**

the specification of which is attached hereto unless the following box is checked.

(x) was filed on **November 8, 1999**  
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or PCT International Application Number  
**PCT/IT99/00355**  
and was amended on  
(if applicable)

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Prior foreign applications  
Domande all'estero precedenti

Priority claimed  
Priorità rivendicata

(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	(X)	(...)	(...)	(...)
BE 988 306 74.2	EPO	9 NOVEMBER 1998	(X)	(...)	(...)	(...)
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	(...)	(...)	(...)	(...)
(Number) (Numero)	(Country) (Paese)	(Day,Month,Year Filed) (Giorno, Mese, Anno di Deposito)	Yes	No	Yes	No
			(...)	(...)	(...)	(...)
			Yes	No	Yes	No

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(Application Serial No.) Numero di domanda	(Filing Date) (Data di deposito)	(Stato Giuridico) (concessa, pendente, abbandonata)	(Legal Status) (patented, pending,

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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SAMVELS, GAUTHIER & STEVENS, LLP  
225 Franklin Street, Suite 3300  
Boston, Massachusetts 02110

Send correspondence to:

Telefonare a: (Nome e Numero)

Tel.: (617) 426 - 9180  
Fax.: (617) 426 - 2275

Direct telephone calls to: (name and telephone number):

Nome completo dell'inventore primo o unico	Full name of sole or first inventor	
Firma dell'inventore	Inventor's signature	Date
Residenza	Residence	
Cittadinanza	Citizenship	
Recapito o Casella Postale	Post Office Address	
Nome completo del secondo inventore, se esistente	Full name of second joint inventor, if applicable	
Firma dell'inventore	Inventor's signature	Date
Residenza	Residence	
Cittadinanza	Citizenship	
Recapito o Casella Postale	Post Office Address	
Nome completo del terzo inventore, se esistente	Full name of third joint inventor, if applicable	
Firma dell'inventore	Inventor's signature	Date
Residenza	Residence	
Cittadinanza	Citizenship	
Recapito o Casella Postale	Post Office Address	

(Si prega di fornire le stesse informazioni e firme di eventuali terzi e più coinventori)

(Supply similar information and signature for third and subsequent joint inventors)

09/831577

SEQUENCE LISTING

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Consiglio Nazionale delle Ricerche  
Spina, Angelo  
Defez, Roberto

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nitrogen fixation and plant biomass production

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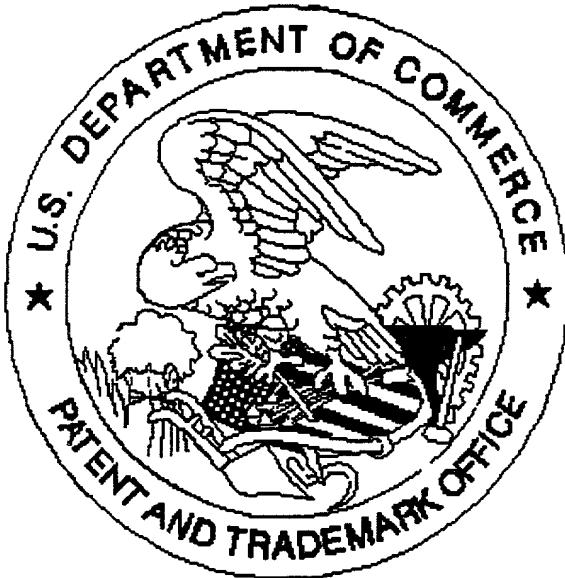
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United States Patent & Trademark Office  
Office of Initial Patent Examination -- Scanning Division



Application deficiencies found during scanning:

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for scanning. (Document title)

Page(s) \_\_\_\_\_ of \_\_\_\_\_ were not present  
for scanning. (Document title)

\* *Scanned copy is best available. Drawing Fig 3, 4, 5, 9, 11,  
are very dark*